IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	ATTY.'S DOCKET: FISHMAN=19A
	)	
FISHMAN ET AL.	)	Art Unit: 1623
	)	
Appln. No.: 10/565,161	)	Examiner: Michael C. Henry
	)	H.
Filed: JANUARY 19 2006	)	
	)	
For: TREATMENT OF INFLAMMATION	)	Confirmation No. 1169

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner for Patents
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Alexandria, VA 22314

Sir:

I, Bruce N. Cronstein, a USA citizen residing at 120 west 88th St., New York, NY 10024, USA, do hereby state and declare as follows:

1. I received my Medical Education from the University of Cincinatti and the Board certifications from the National Board of Medical Examiners (1977) and the American Board of Internal Medicine in Internal Medicine (1980) and Rheumatology (1984).

2. I am currently a Director of the Division of Clinical Pharmacology and Associate Chairman of Medicine for Research in the NYU Medical Center and a member of the Ethics Committee at the American College of Rheumatology.

4. I am also an author / co-author of over 100 scientific publications and books and of 8 US patents or patent applications.

5. My education and professional experience is provided in the attached *Curriculum Vitae* (**Annex A**)

6. My major research interests are focused on the regulation of the inflammatory response and the role of Adenosine-mediated regulation of inflammation, wound healing, fibrosis and bone resorption and the pharmacology of adenosine receptors.

7. Among other research projects, I am involved in exploring the molecular action of methotrexate (MTX) in inflammatory disease, in particular, rheumatoid arthritis and on March 19, 2002 I have co-authored a Review in the matter (Chan ES; Cronstein BN. "Molecular action of methotrexate in inflammatory diseases". Arthritis *research*. 2002; 4:266; hereinafter referred to as the ***"2002 Review"***, a copy of which is attached as **Annex B**), which summarized findings that appeared in a previous research publication:

Morabito L, Montesinos MC, Schreibman DM, Balter L, Thompson LF, Resta R, Carlin G, Huie MA, Cronstein BN. Methotrexate and Sulfasalazine Promotes Adenosine Release by a Mechanism that Requires Ecto-5'-nucleotidase-mediated Conversion of Adenine Nucleotides. *J. Clin. Invest.* 101:295-300, 1998.

Montesinos MC, Yap JS, Desai A, Posadas I, McCrary CT and Cronstein BN. Reversal of the anti-inflammatory effects of methotrexate by the nonselective adenosine receptor antagonists theophylline and caffeine. *Arth. and Rheum.* 43:656-663, 2000.

Montesinos, CM, Desai A, Delano D, Chen JF, Jacobson M, Schwarzschild MA, Fink JS, and Cronstein BN. Bothe adenosine A2A and A3 Receptors are required for inhibition of inflammation by methotrexate and its analogue MX-68. *Arth. Rheum.* 48:240-247, 2003, which was followed by several updated publications discussing the effect of MTX on Rheumatoid Arthritis (RA).

Edwin SL Chan, Patricia Fernandez and Bruce N Cronstein. Methotrexate in rheumatoid arthritis. *Future Drugs Ltd: Expert Review of Clinical Immunology. Volume 3, No. 1, 27-33, January 2007.*

Cronstein BN. Adenosine and Inflammation. *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry* vol. 7, Issue 4, August 2007.

8. The 2002 Review discusses in detail the adenosine-mediated anti-inflammatory effect of MTX. Specifically, this 2002 Review described findings from my laboratory showing that MTX induces metabolic changes which lead to increased extracellular adenosine concentrations (Figure 1 of the 2002 Review - Annex B). Based on the findings in my laboratory, as first published on the findings in Cronstein BN, Eberle MA, Gruber H, Levin RI. Methotrexate inhibits neutrophils function by stimulating adenosine release from connective tissue cells. *Proc. Natl. Acad. Sci.* 88:2441-2445, 1991 and Cronstein BN, Naime D. Ostad E, The anti-inflammatory mechanism of methotrexate: Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J. Clin. Invest.* 92:2675-2682, 1993, it was concluded that adenosine is a key mediator of the anti-inflammatory actions of MTX. In exerting the anti-inflammatory effect the adenosine that

accumulates in the extracellular space upon MTX treatment exerts its anti-inflammatory effect, among others, through the A3 adenosine receptor (A3AR).

9. I have carefully read the above-referenced patent application (serial number 10/565,161; herein: ***"the '161 Application"***). The *'161 application* discloses that the effect of a combined treatment of MTX and an A3AR agonist, CF101.

10. Specifically, the *'161 Application* discloses some *in vivo* studies in an animal model of rats inoculated with heat killed Mycobacterium tuberculosis (Mt). Starting from the 14th days following inoculation, the rats were treated with MTX (intraperitoneally, every three days after inoculation) in combination with IB-MECA (orally, twice a day) or with a control. Clinical Disease Activity Score was assessed, and the results presented in Figures 1A-1B of the '161 Application clearly demonstrate that the combined treatment had an anti-inflammatory effect greater than that of treatment of each agent alone.

11. Being fully conversant with inflammatory reactions, and adenosine-mediated regulation of inflammation, it is my professional opinion that at the time the present patent application was filed there was no *a priori* reason to expect that the addition of an A3AR agonist onto a background of an MTX treatment would exert an anti-inflammatory effect beyond that of MTX alone. There was no publication I am aware of that mentioned such an effect or that could have led someone to suspect that MTX and an A3AR agonists such as IB-MECA would lead to a greater anti-inflammatory effect than each of these agents alone (Montesinos, CM, Desai A, Delano D, Chen JF,

Jacobson M, Schwarzschild MA, Fink JS, and Cronstein BN. Bothe adenosine A2A and A3 Receptors are required for inhibition of inflammation by methotrexate and its analogue MX-68. *Arth. Rheum.* 48:240-247, 2003).

12. In fact, I believe that my own research that showed that the anti-inflammatory effect of MTX is mediated, among others, through the A3AR could have led someone to reach an opposite conclusion that the combined effect of MTX and an A3AR agonist such as IB-MECA, would be no different than each of these agents by themselves since the effect of methotrexate in the previously studied animal modles appeared to be maximal and to have required full engagement of adenosine A3ARs (Montesinos, CM, Desai A, Delano D, Chen JF, Jacobson M, Schwarzschild MA, Fink JS, and Cronstein BN. Bothe adenosine A2A and A3 Receptors are required for inhibition of inflammation by methotrexate and its analogue MX-68. *Arth. Rheum.* 48:240-247, 2003).

13. I herby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 81 of the united states code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

/Bruce Cronstein/ /6/24/08/

Name Date

BRUCE N. CRONSTEIN, MD

Date of birth: May 24, 1951

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Address:
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Education:
1972 Lake Forest College, Lake Forest, Illinois, BA
1976 University of Cincinnati College of Medicine, Cincinnati, Ohio, MD

Professional Experience and Positions:
1976-77 Internship, Internal Medicine, University of Cincinnati Medical Center
1977-78 Resident, Pathology, NYU Medical Center
1978-80 Resident, Internal Medicine, Lenox Hill Hospital, New York
1980-81 Fellow, Rheumatology, NYU Medical Center
1981-82 Chief Fellow, Rheumatology, NYU Medical Center
1982-85 Instructor of Experimental Medicine, NYU Medical Center
1985-92 Assistant Professor of Medicine, NYU Medical Center
1986-01 Director, Arthritis Clinic, Bellevue Hospital
1992-96 Associate Professor of Medicine, NYU Medical Center
1995-01 Director of Rheumatology, Bellevue Hospital
1996- Professor of Medicine and Pathology, NYU Medical Center
2000- Associate Director of the Department of Medicine for Research
2000- Director, Division of Clinical and Molecular Pharmacology
2001- Associate Director, General Clinical Research Center
2003-4 Acting Director, General Clinical Research Center
2005- Director, Masters in Clinical Investigation Training Program, NYU School of Medicine
2006- Program Director, General Clinical Research Center
2006- Director, Clinical and Translational Science Center

Board Certifications:
1977 National Board of Medical Examiners
1980 American Board of Internal Medicine, Internal Medicine
1984 American Board of Internal Medicine, Rheumatology

Fellowships and Awards:

1984-87 Fellow of the Arthritis Foundation
1985-90 Clinical Investigator Award, National Institutes of Health
1985 Travel Award, Arthritis Foundation
1988-91 Irene Duggan Arthritis Investigator Award, Arthritis Foundation
1989 Whitehead Presidential Fellowship, New York University
2000 Alpha Omega Alpha, Honor Medical Society

Hospital Affiliations:

Attending, Bellevue Hospital Medical Center
Attending, NYU Medical Center

Societies:

American Association for the Advancement of Science
American College of Rheumatology
The Harvey Society
New York Rheumatism Association
American Federation for Clinical Research
American Association of Immunologists
American Society for Clinical Investigation
Interurban Clinical Club
American Society for Pharmacology and Experimental Therapeutics
Wound Healing Society
American Society of Investigative Pathology

Boards and Committees :

1997- Advisory Editorial Board, Arthritis and Rheumatism
1987-92 Member, Scientific Advisory Board, Gensia Pharmaceuticals, San Diego, CA.
1988-91 Member, Grant Review Committee, NY Arthritis Foundation
1989-94 Executive Board, New York Rheumatism Association.
1991-95; 98- Medical and Scientific Committee, NY Arthritis Foundation
1992-95 Committee for the Publication of Arthritis and Rheumatism, American College of Rheumatology.
1992-93; 95; 97; 99; 2000 Chairman, Inflammation Subsection, American College of Rheumatology, National Meeting Program Committee
1992-94 Chairman, Grant Review Committee, NY Arthritis Foundation
1993-94 President, New York Rheumatism Association
1995-00 Member, Medical and Scientific Committee, SLE Foundation
1995-96; 98- Member, Cell Biology Study Section, Arthritis Foundation Grant Review Committee
1997- Member, Editorial Board of Clinical and Experimental Rheumatology
1999- Chief Editor, Current Rheumatology Reports
2000- Editor-in-Chief, RheumatologyWeb.com
2000- Chairman, Medical and Scientific Committee, SLE Foundation
1999- NIH, SBIR Study Section

2000- NIH, COBRE Study Section
2000- Editor-in-Chief, Inflammation
2000-04 Research Committee, American College of Rheumatology
2000-04 Professional Meetings Committee, American College of Rheumatology
2001- Member, Editorial Advisory Board, Journal of Pharmacology and Experimental Therapeutics
2001- NIH, GMA-1 Study Section, Permanent ad hoc member
2001-03 Member, VA Merit Review, Immunology Study Section
2003 Chairman, VA Merit Review, Immunology Study Section
2003-06 Chairman, ACTS (formerly GMA-1) Study Section
2005-08 Member, Ethics Committee, American College of Rheumatology

NYU Committees:

1993- General Clinical Research Center, Executive Advisory Committee
1995- Institutional Animal Care and Use Committee
1997-99 Research Space and Policy Committee
1998- Advisory Committee of the Research Computing Resource
2000-01 Departmental Review Policy Committee
2000- Conflict of Interest Committee
2000- Department of Medicine Promotions and Tenure Committee
2002- Chairman, Conflict of Interest Committee
2002-03 Department of Dermatology, Departmental Review Committee

Patents:

1999 Patent number 5,932,558 Adenosine receptor agonists for the promotion of wound healing
2000 Patent number 6,020,321 Adenosine receptor agonists for the promotion of wound healing
2001 Patent number 6,313,091 Pharmaceutical compositions containing TSG-6 for treating inflammatory diseases and cancer-related pathologies
2003 Patent number 6,555,545 Adenosine A_{2A} receptor antagonists for treating and preventing hepatic fibrosis, cirrhosis and fatty liver
2006 Patent pending, Adenosine A_1 receptor antagonists for the treatment of osteoporosis
2006 Patent pending, Testing for single nucleotide polymorphisms in the adenosine A_1 receptor in patients with fibromyalgia
2007 Patent pending, Adenosine A_{2A} receptor agonists for the prevention of prosthesis loosening
2007 Patent pending, Adenosine A_1 and A_{2B} receptor antagonists for the treatment of fatty liver

Major Research Interests:

Regulation of the inflammatory response.
Role of Adenosine and Adenosine receptors in Health and Disease

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51. Montesinos MC, Desai A, Delano D, Chen J-F, Fink JS, Jacobson MA and **Cronstein BN**. Adenosine A_{2A} or A_3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. *Arth.Rheum*. 48:240-247, 2003.
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Review

Molecular action of methotrexate in inflammatory diseases

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Abstract

Despite the recent introduction of biological response modifiers and potent new small-molecule antirheumatic drugs, the efficacy of methotrexate is nearly unsurpassed in the treatment of inflammatory arthritis. Although methotrexate was first introduced as an antiproliferative agent that inhibits the synthesis of purines and pyrimidines for the therapy of malignancies, it is now clear that many of the anti-inflammatory effects of methotrexate are mediated by adenosine. This nucleoside, acting at one or more of its receptors, is a potent endogenous anti-inflammatory mediator. In confirmation of this mechanism of action, recent studies in both animals and patients suggest that adenosine-receptor antagonists, among which is caffeine, reverse or prevent the anti-inflammatory effects of methotrexate.

Keywords: adenosine receptor, inflammation, methotrexate, rheumatoid arthritis

Introduction

The demonstration in 1985 that low-dose, intermittent methotrexate is a potent and effective therapy for rheumatoid arthritis (RA) [1] led to a dramatic change in the way that patients with RA are treated. Indeed, methotrexate is no less efficacious than specific anti-tumor-necrosis-factor (anti-TNF) therapy for the relief of symptomatic joint inflammation in early RA, and the difference between methotrexate and etanercept with respect to protection from structural injury in RA is probably not biologically significant [2]. Thus, methotrexate remains the cornerstone of therapy for RA, and understanding the mechanism(s) responsible for the therapeutic efficacy of this agent may lead to the development of new therapies.

History and clinical pharmacology

Methotrexate was first developed in the 1940s as a specific antagonist of folic acid. This drug inhibits the proliferation of malignant cells, primarily by inhibiting the *de novo* synthesis of purines and pyrimidines. Because administration of high doses of reduced folic acid (folinic acid) or even folic acid itself can reverse the antiproliferative effects of methotrexate, it is clear that methotrexate does act as an antifolate agent. Interestingly, although not originally designed as such, methotrexate appears to be a 'pro-drug', i.e. a compound that is converted to the active agent after uptake. Methotrexate is taken up by cells via the reduced folate carrier and then is converted within the cells to polyglutamates [3]. Methotrexate polyglutamates are long-lived metabolites that retain some of the antifolate activities of the parent compound, although the potency for inhibition of various folate-dependent enzymes is shifted [3–6].

Proposed mechanisms of action of methotrexate

Low-dose methotrexate was introduced for the treatment of RA because of its presumed antiproliferative properties, although it was unclear how inhibiting proliferation of the lymphocytes thought to be responsible for synovial inflammation in RA for one day a week might lead to effective suppression of disease activity. However, it soon became clear that inhibition of folic acid metabolism could not be completely responsible for the anti-inflammatory effect of methotrexate. During the past 15 years, it has become clear that administration of folic acid in doses of 1–5 mg per day helps to prevent much of the toxicity of methotrexate without interfering with the anti-inflammatory efficacy of the drug, whereas very high doses of folinic acid also prevent methotrexate toxicity but may interfere with its efficacy [7–20]. There are two potential explanations for the

AICAR = aminoimidazolecarboxamidoribonucleotide; Fc = crystallizable fragment (of antibody); IFN = interferon; IL = interleukin; RA = rheumatoid arthritis; Th = T helper (cells); TNF = tumor necrosis factor.

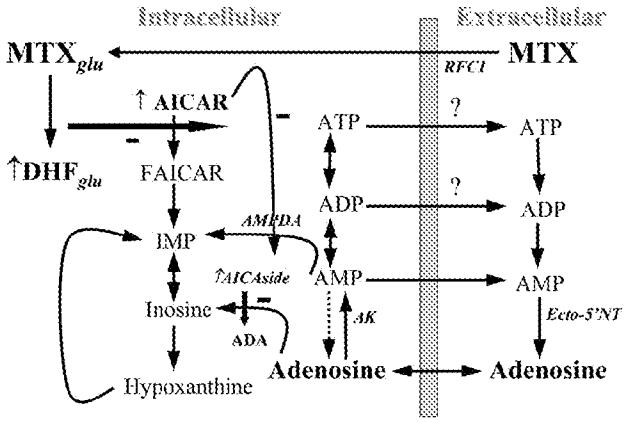
capacity of high doses of folinic acid to reverse the therapeutic effects: first, folinic acid may bypass the effects of methotrexate on reduction of folic acid and thereby bypass the therapeutic effects of the drug; alternatively, folinic acid but not folic acid may compete with methotrexate for a single transport site into the cell (Fig. 1) and may thus interfere with cellular uptake of methotrexate [21]. Moreover, the expected inhibition of cellular proliferation is manifested as bone marrow suppression, and oral and gastrointestinal ulcers, and may require lowering the dose of the drug and, usually, the efficacy of the therapy, suggesting that inhibition of cellular proliferation alone is not responsible for the anti-inflammatory effects of methotrexate. Thus, folate antagonism appears to play, at most, a minimal role in the anti-inflammatory mechanism of methotrexate.

Another potential mechanism by which methotrexate may diminish inflammation in the joint is by diminishing cytokine production. Numerous studies have demonstrated diminished levels of inflammatory cytokines in the serum of patients. The adenosine A_{2A} receptor agonist CGS-21680 is a potent inhibitor of neutrophil leukotriene synthesis *in vitro,* and, similarly, methotrexate therapy leads to diminished production of leukotriene B_4 by neutrophils stimulated *ex vivo* [22,23]. The mechanism by which methotrexate diminishes these cytokine levels remains unexplained and it is difficult to determine from these studies whether the effects of methotrexate therapy on production of inflammatory mediators results in diminished inflammation or is secondary to other anti-inflammatory events.

Similarly, methotrexate-mediated effects on T-cell function, either *in vivo* or *in vitro,* have been demonstrated. Indeed, Genestier and colleagues have reported that methotrexate diminishes antigen-stimulated T-cell proliferation both *in vitro* and in T cells taken from patients taking methotrexate [24]. That the effects of methotrexate on T-cell function are completely reversed by folic acid and that the effects of therapy on T cells studied *ex vivo* are present for only 48 hours a week would strongly suggest that this cannot be responsible for the bulk of the anti-inflammatory effects of the drug.

A third proposed mechanism of action is based upon the observation that polyamines accumulate in the synovium of patients with RA and that metabolism of these polyamines by macrophages leads to the production of toxic oxygen products that diminish stimulated T-cell function [25–27]. Indeed, methotrexate therapy does diminish polyamine levels in the joints of patients with RA [28–30], but this effect, like that of methotrexate on T-cell proliferation, is reversed by folic acid. Moreover, there are more than enough toxic oxygen metabolites being generated in the rheumatoid synovium to mediate the tissue damage present in this disease; another source of toxic agents would add relatively little.

Figure 1



Methotrexate-induced metabolic changes lead to increased extracellular adenosine. ADA = adenosine deaminase; AICAR = aminoimidazolecarboxamidoribonucleotide; AICAside = aminoimidazolecarboxamidoribonucleoside; AK = adenosine kinase; AMPDA = AMP deaminase; DHF = dihydrofolate; DHF_{glu} = dihydrofolate polyglutamate; ecto-5′NT = ecto-5′nucleotidase; FAICAR = formyl-AICAR; IMP = inosine monophosphate; MTX = methotrexate; MTX_{glu} = methotrexate polyglutamate; RFC1 = reduced folate carrier 1.

Methotrexate induces adenosine release

Our laboratory originally proposed the hypothesis that the beneficial effects of methotrexate result from the intracellular accumulation of intermediates in purine biosynthesis that, by a mechanism that has not been completely worked out, leads to increased concentrations of adenosine in the extracellular space [31]. This hypothesis sprang from the prior demonstration that intracellular accumulation of specific intermediates in the *de novo* synthesis of purines leads to adenosine release [32] and from our interest in the anti-inflammatory effects of adenosine, which are mediated by specific receptors on inflammatory cells. Prior work had demonstrated that methotrexate polyglutamates inhibit the enzyme aminoimidazolecarboxamidoadenosineribonucleotide (AICAR) transformylase more potently than the other enzymes involved in purine biosynthesis [4,5,33]. This inhibition occurred at pharmacologically relevant concentrations of methotrexate and might be expected to occur more readily with infrequent loading with methotrexate, since methotrexate polyglutamates are long-lived metabolites (persisting for weeks). The presence of increased concentrations of AICAR metabolites in the urine of RA patients treated with methotrexate supports these findings [34,35]. The accumulation of AICAR and its metabolites has a direct inhibitory effect on at least two key enzymes, adenosine deaminase and AMP deaminase, with the end result of increased concentrations of adenosine and adenine nucleotides intracellularly [4]. Methotrexate in doses similar to that used in the treatment of RA has been known

to cause the accumulation of AICAR in animal models of RA, and this accumulation is associated with an elevation in adenosine concentration in the extracellular space [32,36]. The exact mechanisms by which the elevation of extracellular adenosine arises are not fully understood, but dephosphorylation of adenine nucleotides is likely to be a major contributor, partly because of the ubiquitous nature of ATP in tissues and partly because of the widespread existence of ecto-5′-nucleotidase, an enzyme that catalyzes the dephosphorylation of AMP to adenosine [37].

All this evidence points to adenosine as a key mediator in the anti-inflammatory actions of methotrexate. *In vivo* experiments support this contention. The nonselective adenosine receptor antagonist 8-phenyl theophylline potentiated inflammatory responses in a hamster-cheek-pouch model [38]. Infusion of adenosine directly into the knee in rats inhibited the development of adjuvant-induced arthritis, and an adenosine receptor antagonist effectively reduced the severity of joint inflammation in a collagen-induced arthritis model in mice [39,40]. We have previously shown that the anti-inflammatory effects of methotrexate in carrageenan-induced mouse air pouch inflammation is reversed by an antagonist to the adenosine A_{2A} receptor, or by the addition of adenosine deaminase, an adenosine-metabolizing enzyme, suggesting that adenosine is indeed responsible for the anti-inflammatory effects of methotrexate *in vivo* [36]. An interesting study by Silke *et al.* showed that ingestion of caffeine, a nonselective antagonist of adenosine receptors, in coffee correlates with poor clinical response to methotrexate, and patients with a high caffeine intake are more likely to discontinue methotrexate than those with a low caffeine intake [41].

To better appreciate how adenosine influences biological responses in the network of events taking place in an inflammatory milieu, something must be said about this autocoid and the cellular receptors with which it interacts to produce these physiological responses. Adenosine receptors, or P1 receptors, fall into four known subclasses: A_1, A_{2A}, A_{2B}, and A_3. These are members of the large, seven-transmembrane-receptor family of receptors that influence cell signaling mechanisms by coupling to G proteins. The receptor sequences have been characterized and, with the exception of the A_3 receptor, they are highly conserved during evolution. Adenosine receptors modulate a vast array of physiological functions, from heart rate to the state of wakefulness. Adenosine, acting on P1 receptors, exerts a number of actions on a variety of cell types relevant to the anti-inflammatory effect of methotrexate.

Cellular effects

Neutrophils

Neutrophils, a hallmark of acute inflammation, are among the first cells recruited into the inflammatory site. The limitation of neutrophilic-mediated damage relies in part on the modification of the adhesive capacity and ability to generate chemical damage, properties under purinergic influence. The resting neutrophil has a number of mechanisms that, once activated, can damage tissues. One of these is latent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multimolecular complex that is assembled at the plasma membrane upon activation of the neutrophil and that generates oxygen radicals [42]. The first in the chain of these oxygen radicals is superoxide anion, and it was the discovery in 1983 that superoxide generation, as stimulated by a variety of agents including the chemoattractant *N*-formyl-leucyl-phenylalanine (*f*MLP), the complement component C5a, and the calcium ionophore A23187, was inhibited by adenosine that sparked an interest in the anti-inflammatory properties of adenosine [43,44]. This physiological action of adenosine has subsequently been ascribed to its action on the adenosine A_{2A} receptor, which is present on the neutrophilic surface membrane [45]. An important second messenger to adenosine-A_{2A}-receptor signaling in this respect appears to be 3′,5′-cyclic adenosine monophosphate (cAMP), the intracellular concentration of which increases with neutrophilic adenosine A_{2A} receptor stimulation. cAMP further activates protein kinase A downstream and inhibition of protein kinase A reverses the effects of cAMP analogues but not of adenosine receptor agonists on stimulated neutrophilic superoxide anion generation [46]. The cAMP–protein-kinase-A-dependent adenosine inhibition of neutrophil oxidative activity is mediated via the adenosine A_{2A} receptor [47]. One direct consequence of the interruption of superoxide anion formation and respiratory burst reactions is the protection of vascular endothelial cells from neutrophil-mediated injury [48].

The adenosine-A_{2A}-receptor-mediated effects on neutrophil function are dose-related. At concentrations similar to those required to inhibit the release of superoxide anions, adenosine, acting through A_{2A} receptors, inhibits adherence to endothelial cells by stimulated neutrophils [49]. This may be related in part to dose-related preferential recruitment of receptor subtype, since the adenosine A_1 receptor exhibits many opposing physiological functions to those mediated by the A_{2A} receptor, including stimulation of neutrophil adherence to endothelial cells. Adenosine also inhibits the release of vascular endothelial growth factor from neutrophils, thereby enhancing vascular permeability [50]. The dose-dependent response in adenosine action is also seen with Fc-gamma-receptor-mediated neutrophil phagocytosis, which is enhanced by A_1 receptor stimulation but inhibited via A_2 receptors [51]. In addition, adenosine also inhibits the TNF-induced generation of elastase by neutrophils [52].

Expression of adhesive molecules is an important event that guides neutrophil recruitment into an inflammatory site through adhesion to the vascular endothelium.

Adenosine has been known to be a modulator of the expression or function of adhesive molecules including β_2-integrin, L-selectin, and CD11b/CD18 [49,53,54]. The activity of adenosine in the modulation of neutrophil adhesion again demonstrates the opposing roles of A_1 and A_2 receptors [49].

Macrophages

Cells of the monocyte–macrophage series are abundant in the rheumatoid synovium and pannus and contribute significantly to the tissue damage seen in both acute and chronic disease, as recently reviewed by Kinne and colleagues [55]. Macrophages, the differentiated tissue form, are also critical producers of cytokines that play a prominent role in promoting proinflammatory responses that culminate in tissue damage. Like neutrophils, their capacity to phagocytose opsonized particles and to generate superoxide anions plays a major role in eliciting tissue damage. Inhibition of Fc-gamma-receptor phagocytic activity in cultured monocytes is exhibited by adenosine at high concentrations such as that seen with tissue damage and is a function mediated via adenosine A_2 receptors, while low concentrations of adenosine have the opposite effect on Fc-gamma-receptor phagocytic activity mediated via adenosine A_1 receptors [56]. Similarly, adenosine inhibits the generation of superoxide anions by monocytes stimulated with *N*-formyl-leucyl phenylalanine [57].

One of the well known though uncommon side effects of methotrexate treatment is the formation of subcutaneous nodules, often similar in histological appearance though not in distribution to those found in rheumatoid disease. A hallmark of these subcutaneous nodules is the existence of the multinucleated giant cell, formed by fusion of macrophages. The fusion of macrophages into multinucleated giant cells is enhanced by stimulation of the adenosine A_1 receptor and is inhibited by activation of the A_2 receptor [58,59].

The recent success of anti-TNF therapy highlights the role of cytokines as important mediators of inflammatory activity. Not surprisingly, methotrexate, still one of the most effective disease-modifying antirheumatic drugs for the treatment of RA, acting through the release of adenosine, also inhibits the production of TNF-α, although the adenosine receptor involved in this action remains controversial [60–63]. Modulation of cytokine production by adenosine extends far beyond TNF-α and includes observable effects on IL-6, IL-8, IL-10, IL-12, and macrophage inflammatory protein-1α (MIP-1α) [40,64,65]. Cytokines themselves can regulate the expression of adenosine receptors on monocytic cells and thereby modulate adenosine-mediated responses, as we and others have recently shown [66,67]. Macrophage production of nitric oxide and nitric oxide synthase is also inhibited by adenosine, probably via A_{2B} receptors [65,67].

Endothelial cells

Endothelial cells are effective transit barriers between vessels and tissue and as such are notable in inflammation not only because of their expression of adhesive molecules, which allow leukocytes their access to inflammatory sites. The effectiveness of this barrier function relies in part on the preservation of impermeability to circulating cells homing in to take part in inflammatory reactions in the tissues. Adenosine enhances this barrier function by decreasing enthothelial permeability via A_{2B} receptor and helps limit potential tissue damage [68,69]. Production of inflammatory cytokines such as IL-6 and IL-8 and expression of adhesive molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin by endothelial cells are also suppressed by adenosine [70]. Another important aspect of inflammation lies in the proliferation and migration of endothelial cells in the process of angiogenesis, which is enhanced by the presence of adenosine, probably acting through A_2 receptors [71–73]. Adenosine may also induce apoptosis of endothelial cells, thus potentially enhancing the extravasation of inflammatory fluids [74].

Humoral and cellular immune responses

Rheumatoid factor, or autoantibodies directed against the Fc portion of IgG, is a hallmark of RA, although its exact role in the pathogenesis of the disease has been debated. The effect of methotrexate on the levels of circulating IgM rheumatoid factors has also been controversial. While some workers have reported no suppression of serum rheumatoid factor levels with methotrexate treatment, Alarcon *et al.* observed significant drops in the levels of both IgM and IgA rheumatoid factors in methotrexate-treated patients, and particularly of the concentration of IgM rheumatoid factor in those who showed clinical improvement [75]. These findings were confirmed by other groups in studies done both *in vivo* and *ex vivo* [76–80], although it is unclear whether this is a primary or secondary effect of adenosine.

T lymphocytes have received much attention in relation to the pathogenesis of RA and opinions differ in their contribution to the causation of the disease. The presence of these cells in the affected synovium and the strong ethnicity-dependent HLA–DR associations implicate T lymphocytes as key players in the disease process. One possible explanation of the beneficial actions of methotrexate in RA is the diminution of both the size and reactivity of the T-lymphocyte population. There are suggestions that this may be accomplished by the induction of apoptosis in activated T cells [24]. This suggestion is consistent with the observations of reductions in peripheral blood T and B lymphocyte populations after short-term methotrexate treatment [81], and methotrexate induction of apoptosis in inflammatory cells may be relevant to its antirheumatic actions *in vivo* [82]. In contrast, significant increases in the CD3- and CD4-positive peripheral blood cells and

enhancement of stimulated lymphocyte proliferation have been observed after long-term treatment with methotrexate [83], and adenosine, acting through A_{2A} and A_{2B} receptors, may play a role in T-cell deactivation [84,85]. Nonetheless, the role of these shifts in T-cell function and trafficking in the pathogenesis of RA is unclear.

Phlogistic responses

Cytokines are messengers with major roles in inflammatory and immune responses and have been targets of interest in recent therapeutic developments in chronic arthritis, with TNF-α and IL-1 as the focus of interest [86]. In animal models of chronic arthritis, methotrexate was thought to be useful in reducing the production of IL-1 [87,88]. In support of these findings, clinical studies of RA patients receiving methotrexate treatment have documented reductions in monocytic IL-1 production but not serum concentrations of IL-1 [89]. Others have disputed this view and suggested that alterations in IL-1 responses were related to diminutions in the ability of cells to respond to IL-1 rather than to direct inhibition of its production, perhaps through dose-dependent ligand binding [90–92].

Methotrexate is also known to suppress TNF activity by suppressing TNF-induced nuclear factor-κB activation *in vitro*, in part related to a reduction in the degradation and inactivation of an inhibitor of this factor, IκBα, and probably related to the release of adenosine [93]. The generation of TNF-α by peripheral blood mononuclear cells is suppressed by an adenosine kinase inhibitor, by virtue of its ability to limit adenosine uptake and metabolism and thereby enhance extracellular adenosine concentration [94]. TNF-α synthesis in T cells and macrophages is suppressed [95]. In the murine collagen-induced arthritis model, *in vivo* intraperitoneal methotrexate treatment reduced TNF serum levels and diminished TNF production by splenic T cells and macrophages [96]. Methotrexate suppresses the production of both TNF and IFN-γ by T-cell-receptor-primed T lymphocytes from both healthy human donors and RA patients [97]. In early RA, in which the disease duration is less than 6 months, methotrexate treatment is associated with a significant decrease of TNF-α-positive $CD4^+$ T cells, while the number of T cells expressing the anti-inflammatory cytokine IL-10 increased [98]. Methotrexate is also known to suppress the IL-6-induced generation of reactive oxygen species in the synoviocytes of RA patients [99]. Serum IL-6 levels have also declined after methotrexate treatment in RA patients in some studies [100]. Constantin *et al.* reported that *ex vivo* treatment of peripheral blood monocytes with methotrexate increased expression of IL-4 and IL-10 while IL-2 and interferon-γ expression were decreased, suggesting that the immunoregulatory role of methotrexate is also targeted at adjusting the balance between Th1 proinflammatory and Th2 anti-inflammatory cytokines [101]. Again, the molecular mechanism of these changes is unclear.

Conclusion

Our search for mechanisms governing the inflammatory response has uncovered many facets relevant to the pathogenesis of arthitic diseases. The success of methotrexate as an antirheumatic agent rests on its many actions that affect a wide variety of pathogenic mechanisms, many of which are mediated by the release of adenosine. The molecular mechanism for many of these phenomena is related to the enhanced release of adenosine into the extracellular space, where it can activate its receptors on relevant cell types. In this respect, methotrexate is an excellent example of how knowledge and continuing research in molecular biology and pharmacology can be employed in the refinement of existing medications originally used on an observational basis. Such understanding will form the basis for the development of new and more effective therapy for the treatment of rheumatic diseases.

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Methotrexate and Sulfasalazine Promote Adenosine Release by a Mechanism that Requires Ecto-5′-nucleotidase–mediated Conversion of Adenine Nucleotides

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Abstract

We and others have shown that an increased extracellular concentration of adenosine mediates the antiinflammatory effects of methotrexate and sulfasalazine both in vitro and in vivo, but the mechanism by which these drugs increase extracellular adenosine remains unclear. The results of the experiments reported here provide three distinct lines of evidence that adenosine results from the ecto-5′-nucleotidase–mediated conversion of adenine nucleotides to adenosine. First, pretreatment of a human microvascular endothelial cell line (HMEC-1) with methotrexate increases extracellular adenosine after exposure of the pretreated cells to activated neutrophils; the ecto-5′-nucleotidase inhibitor α,β–methylene adenosine-5′-diphosphate (APCP) abrogates completely the increase in extracellular adenosine. Second, there is no methotrexate-mediated increase in extracellular adenosine concentration in the supernate of cells deficient in ecto-5′-nucleotidase, but there is a marked increase in extracellular adenosine concentration in the supernates of these cells after transfection and surface expression of the enzyme. Finally, as we have shown previously, adenosine mediates the antiinflammatory effects of methotrexate and sulfasalazine in the murine air pouch model of inflammation, and injection of APCP, the ecto-5′-nucleotidase inhibitor, abrogates completely the increase in adenosine and the decrement in inflammation in this in vivo model. These results not only show that ecto-5′-nucleotidase activity is a critical mediator of methotrexate- and sulfasalazine-induced antiinflammatory activity in vitro and in vivo but also indicate that adenine nucleotides, released from cells, are the source of extracellular adenosine. (*J. Clin. Invest.* 1998. 101:295–300.) Key words: adenosine • ecto-5′-nucleotidase • methotrexate • sulfasalazine • inflammation

Introduction

We have demonstrated previously in both in vitro and in vivo studies that the antiinflammatory properties of low-dose methotrexate and sulfasalazine are mediated by adenosine, a potent antiinflammatory autocoid (1–3). Methotrexate and sulfasalazine promote adenosine release from a variety of different cell types and tissues, particularly when the cells or tissues are undergoing a physiologic stress (1, 3). The adenosine released at inflamed sites interacts with specific receptors on inflammatory cells to diminish inflammation and tissue injury (for a review see reference 4).

The mechanism by which methotrexate and sulfasalazine promote adenosine release is not well understood. Both methotrexate and sulfasalazine are taken up by cells where they or their metabolites inhibit 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)[1] transformylase (5–7). The two- to threefold increase in intracellular (splenocyte) AICAR concentration in animals treated with pharmacologically relevant doses of methotrexate or sulfasalazine is consistent with the hypothesis that low-dose methotrexate treatment leads to inhibition of AICAR transformylase in vivo (2, 3). Intracellular AICAR accumulation has been associated with adenosine release (8–10), although how and whether intracellular AICAR accumulation promotes adenosine release have not been established.

One potential explanation for the effect of AICAR accumulation on adenosine release is that AICAR inhibits AMP deaminase, thereby increasing intracellular AMP, which may be dephosphorylated either intracellularly or extracellularly to adenosine. Alternatively, accumulated AICAR may be dephosphorylated to its ribonucleoside, an inhibitor of adenosine deaminase, an enzyme that irreversibly deaminates adenosine and deoxyadenosine to inosine and deoxyinosine.

To begin to understand the molecular mechanism by which methotrexate treatment leads to adenosine release, we tested the hypothesis that the adenosine released from stressed cells and tissues after treatment with methotrexate and sulfasalazine is derived from the extracellular dephosphorylation of adenine nucleotides rather than the direct release of adenosine. We report here evidence from both in vitro and in vivo experiments that the methotrexate- and sulfasalazine-mediated increase in extracellular adenosine is accounted for completely by the extracellular generation of adenosine from adenine nucleotides via the ecto-5′-nucleotidase–catalyzed dephosphorylation of AMP.

Methods

Materials. α,β–Methylene adenosine-5′-diphosphate (APCP) and carrageenan were obtained from Sigma Chemical Co. (St. Louis,

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1. *Abbreviations used in this paper:* AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; APCP, α,β–methylene adenosine-5′-diphosphate; HBS, Hepes-buffered saline; HMEC-1, human microvascular endothelial cell line 1; LDH, lactate dehydrogenase.

MO). All tissue culture media and reagents were purchased from GIBCO BRL (Gaithersburg, MD). Methotrexate was obtained from Immunex Corp. (San Juan, Puerto Rico). All other reagents were of the highest quality obtainable.

Isolation of leukocytes. Human neutrophils were isolated from whole blood after centrifugation through Hypaque-Ficoll gradients, sedimentation through dextran (6% wt/vol), and hypotonic lysis of red blood cells. Neutrophils were suspended in Hepes-buffered saline (HBS) supplemented with Mg^{2+} and Ca^{2+} and counted before addition to confluent human microvascular endothelial cell line 1 (HMEC-1) monolayers (11).

Cell culture. HMEC-1 (obtained from the Centers for Disease Control and Prevention) was cultured in 96-well plates in 200 μl of MCDB 131 supplemented with 10% FBS, 3% 200 mM L-glutamine, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. Subconfluent (60–70% confluent) monolayers were then washed twice with fresh medium and incubated for 48 h at 37°C and 5% CO_2 atmosphere in fresh medium alone or medium containing methotrexate (100 nM). Both methotrexate-treated and control HMEC-1 cells reached confluence under these conditions by the time experiments with neutrophils were performed.

293T cells, a human renal carcinoma cell line transfected with large T antigen, deficient for ecto-5′-nucleotidase (CD73 [12]) were grown to 60–70% confluence (before transfection and treatment with methotrexate) on 6-well plates in 4 ml DME supplemented with 10% FCS, 3% 200 mM L-glutamine, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Monolayers were then washed, and medium was replaced with either fresh medium alone or medium containing methotrexate (100 nM). Cells were then grown to confluence over 48 h. Under these conditions, methotrexate did not diminish cell proliferation, and the 293T cells grew to confluence in medium with or without methotrexate.

Stimulation of confluent HMEC-1 with PMNs in the presence of APCP. Monolayers of HMEC-1 cells grown to confluence in 96-well plates were washed twice with medium. To each well were then added, sequentially, 50 μl of HBS containing APCP (50 μM) or HBS alone, 100 μl of HBS containing 1.5×10^5 PMNs, followed by 50 μl of either HBS alone or HBS containing the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP, 100 nM). Plates were then incubated for 2 h (37°C, 5% CO_2).

Collection of supernatants and pouch exudates for quantitation of adenosine by HPLC. Aliquots of pouch exudates or cell culture supernates were added to a similar volume of TCA (10% vol/vol) followed by extraction of the organic acid with freon/trioctylamine (77.5/22.5 %, vol/vol). The adenosine concentration of the supernatants was determined by reverse-phase HPLC, as we have described previously (13). Briefly, samples were applied to a μBondapackC18 column (Waters Corp., Milford, MA) and eluted with a 0–40% linear gradient (formed over 60 min) of 0.01 M ammonium phosphate (pH 5.5) and methanol, with a 1.5 ml/min flow rate. Adenosine was identified by retention time and the characteristic ultraviolet ratio of absorbance at 250/260, and the concentration was calculated by comparison to standards. In some experiments, the adenosine peak was digested by treatment with adenosine deaminase (0.15 IU/ml, 30 min at 37°C) to confirm that the peak so identified contains only adenosine (14). Preliminary studies demonstrated that 90% of added adenosine is recovered using this technique.

Expression vectors and transfection of CD73 into 293T cells. The $p\beta^{NT}$ expression vector was constructed as described and contains a cDNA for CD73 (15, 16). The empty expression vector, $p\beta^{neo}$, which lacks the CD73 cDNA insert, was used as a negative control (sham transfection). The expression vectors for $p\beta^{NT}$ and $p\beta^{neo}$ were transformed into DaH5 *Escherichia coli*, and positive clones were selected by ampicillin resistance and restriction enzyme digest. Large scale plasmid preparations were grown and purified following the instructions provided in a commercial kit (QIAGEN Inc., Chatsworth, CA). Medium was removed from subconfluent 293T fibroblasts on 6-well plates and replaced with 3.7 ml DME containing either methotrexate (100 nM) or medium alone, supplemented with 10% FCS, 3% 200 mM L-glutamine, and 1% penicillin/streptomycin. After a 4-h incubation at 37°C in a humidified atmosphere of 5% CO_2, cells were transfected with 300 μl of either a $p\beta^{NT}$ or $p\beta^{neo}$ plasmid DNA calcium phosphate solution (5 μg of plasmid per 4 ml of medium) via the calcium phosphate precipitation method (17). This medium was removed after a 12–16-h incubation period and replaced with fresh medium or medium in the presence of methotrexate. Cells were incubated for an additional 24 h, for a total of 44–48 h of methotrexate treatment (15).

Stimulation of 293T fibroblasts with 100 μM hydrogen peroxide, and collection of supernates for adenosine determination. Transfected cells were washed twice with HBS and were then treated with 100 μM H_2O_2 in Hepes or Hepes alone for 1 h at 37°C and 5% CO_2. Supernatants were collected for HPLC analysis as described above. Neither transfection, methotrexate treatment, H_2O_2, nor their combination altered cell viability, as determined by release of lactate dehydrogenase (LDH), as we have described previously (13).

Immunofluorescence. Surface expression of ecto-5′-nucleotidase (CD73) was determined after immunofluorescent labeling by flow cytometry (FACScan®; Becton Dickinson, Mountain View, CA) using techniques we have described previously (18). Briefly, cells were stripped from their substrate after incubation in EDTA (0.01% wt/vol) in PBS followed by scraping with a rubber policeman. Cells were washed and then resuspended ($2–4 \times 10^6$ in a final volume of 50 μl) in PBS or PBS containing 25 μg/ml anti-CD73 (1E9 [15]) or FLOPC-21 (a murine mAb directed against an irrelevant antigen). Labeled cells were then resuspended in 1 ml of PBS/1% sodium azide, washed, and then labeled by incubation in the presence of purified goat anti–mouse IgG_3 labeled with phycoerythrin (Southern Biotechnology Associates, Inc., Birmingham, AL). Labeling was quantitated by flow cytometry (FACScan®).

Carrageenan-induced inflammation in the murine air pouch. Mice (BALB/c; Taconic Farms Inc., Germantown, NY) were treated weekly with methotrexate (0.5 mg/kg) or a similar volume of saline intraperitoneally, followed by induction of inflammation (injection of 1 ml of a suspension of carrageenan, 2% wt/vol) in an air pouch developed on the back of the mice, as we have described previously (2). The air pouch exudate was collected, and the number of leukocytes and adenosine concentration were quantitated, as we have described (2). In other experiments, the animals received sulfasalazine (100 mg/kg) or a similar volume of saline daily by gastric gavage during the induction of the air pouch for 3 d. On the third day, inflammation was induced by injection of carrageenan (3).

Statistical analysis. Data were analyzed using a two-tailed Student's *t* test with the statistical package included in EXCEL (Microsoft, Inc., Redmond, WA).

Results

To determine whether adenosine is generated extracellularly from nucleotides or is released from an intracellular store, we determined the effect of the ecto-5′-nucleotidase inhibitor APCP (50 μM) on adenosine release from HMEC-1 cells exposed to stimulated neutrophils. We found, as we have reported previously for fibroblasts and human umbilical vein endothelial cells, that stimulated neutrophils promote adenosine release from methotrexate-treated HMEC-1 (Fig. 1; $P < 0.01$, $n = 6$). The ecto-5′-nucleotidase inhibitor APCP did not decrease significantly adenosine concentrations in supernates of control monolayers but abrogated completely the methotrexate-induced adenosine increase in supernates of methotrexate-treated HMEC-1 cells ($P < 0.01$, $n = 6$). These findings are consistent with the hypothesis that the increased extracellular adenosine concentration present in supernatants of methotrexate-treated HMEC-1 cells is derived from nucleotides released into the extracellular space.

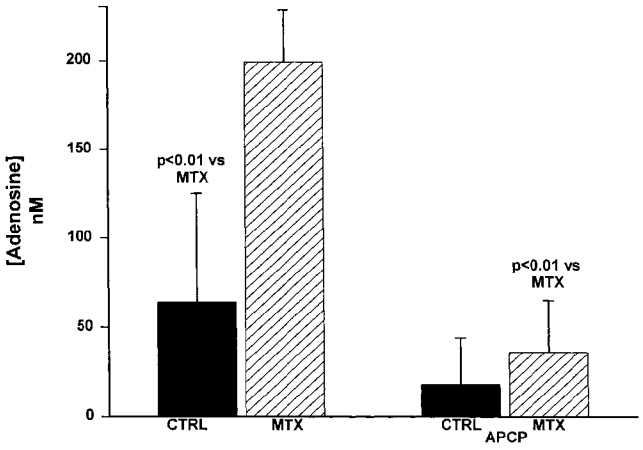

Figure 1. The effect of the ecto-5′-nucleotidase inhibitor APCP (50 μM) on methotrexate (*MTX*)-mediated increases in extracellular adenosine. Subconfluent monolayers of the HMEC-1 were incubated with medium alone (*CTRL*) or medium plus methotrexate (0.1 μM) for 48 h (37°C, 5% CO_2) until confluent. After washing, the monolayers were then exposed to stimulated (FMLP, 100 nM) neutrophils (1.5×10^5 per well) for 2 h (37°C, 5% CO_2) before the supernates were collected and adenosine was quantitated, as described. Shown are the results (±SEM) of six experiments.

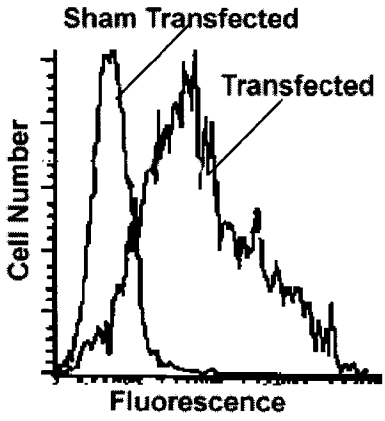

Figure 2. 293T cells transfected with ecto-5′-nucleotidase express the enzyme on their surface. 293T cells transfected with $p\beta^{NT}$ expression vector containing the coding sequence of ecto-5′-nucleotidase (*Transfected*) or with the expression vector alone (*Sham Transfected*) were harvested and labeled for the expression of ecto-5′-nucleotidase by immunofluorescence, as described. Shown is a single representative cytofluorogram (of six) demonstrating surface expression of ecto-5′-nucleotidase on 293T cells. Labeling of sham-transfected cells did not differ from background or isotype control–labeled cells.

To confirm the hypothesis that adenosine is formed extracellularly by the ecto-5′-nucleotidase–mediated dephosphorylation of AMP, we determined the effect of methotrexate on adenosine release from cells deficient in ecto-5′-nucleotidase (293T cells [12]). Because these cells adhere poorly to their substrate after exposure to stimulated neutrophils, a phenomenon which might reflect significant cellular injury, we studied the effect of oxidant stress (H_2O_2, 100 μM), a stress that leaves the monolayers intact, on adenosine release by 293T cells. Supernatants of 293T cells did not contain detectable adenosine, whether the cells were resting, treated with methotrexate, exposed to H_2O_2, or treated with methotrexate followed by H_2O_2 (Table I). After transfection and expression of ecto-5′-nucleotidase (optimal expression at 24 h; Fig. 2), adenosine could be detected only in the supernate of cells pretreated with methotrexate followed by H_2O_2 treatment, but not in sham-transfected cells (Table I). These studies provide more rigorous proof of the hypothesis that adenine nucleotide, released in excess from methotrexate-treated cells, is converted extracellularly to adenosine by the action of ecto-5′-nucleotidase. Moreover, the strong correlation of the results obtained with ecto-5′-nucleotidase–deficient cells with the results obtained using the ecto-5′-nucleotidase inhibitor APCP confirms the selectivity of the inhibitor as far as adenosine production is concerned.

Table I. Adenosine Release by 293T Cells: The Effect of Methotrexate, H_2O_2, and Expression of Ecto-5′-nucleotidase

	Medium	Medium + H_2O_2	Methotrexate	Methotrexate + H_2O_2
No transfection	ND	ND	ND	ND
Sham transfection	ND	ND	ND	ND
Transfection and expression of ecto-5′-nucleotidase	ND	ND	ND	65±6 nM*

293T cells were grown to 60–70% confluence before transfection with $p\beta^{neo}$ expression vector alone (sham transfection) or the $p\beta^{NT}$ vector containing cDNA for CD73, as described. Cells were then cultured for a further 44–48 h in the presence or absence of methotrexate (1 μM) before washing and exposure of the cells to H_2O_2 (100 μM). After 3 h of incubation with medium or H_2O_2, the medium was collected, and the adenosine content was quantitated by HPLC. In these experiments, all conditions were performed in triplicate, and the results shown represent the mean (±SEM) of three different experiments. In parallel experiments, neither methotrexate nor H_2O_2 treatment increased LDH release (< 2% release under all conditions) from control, sham-transfected, or transfected cells. *ND*, None detected. The lower limit of detection is 5 nM. *$P < 0.001$ vs. control, Student's *t* test.

In previous studies, we have demonstrated that methotrexate promotes a marked increase in adenosine release into an inflammatory exudate, and that the increase of adenosine diminishes inflammation (2). To test further the hypothesis that adenosine is derived from the extracellular dephosphorylation of AMP by ecto-5′-nucleotidase, we determined the effect of the ecto-5′-nucleotidase inhibitor APCP on inflammation in the murine air pouch. As we have reported previously, methotrexate treatment promoted adenosine release ($P < 0.01$, $n = 6$) and diminished leukocyte accumulation in the murine air pouch (Fig. 3; $P < 0.05$, $n = 6$). Injection of APCP into the inflamed air pouch did not affect either adenosine release or leukocyte accumulation in control animals or animals treated with dexamethasone (data not shown), but abrogated completely the methotrexate-mediated increase in exudate adenosine concentration ($P < 0.01$, $n = 6$) and decrease in leukocyte accumulation ($P < 0.05$, $n = 6$). These results parallel the results of the in vitro experiments and confirm the hypothesis that methotrexate treatment leads to increased extracellular adenosine concentrations by a mechanism which is dependent upon the extracellular dephosphorylation of adenine nucleotides.

We have demonstrated previously that adenosine also mediates the antiinflammatory effects of sulfasalazine in the murine air pouch model of inflammation (3). Therefore, we deter-

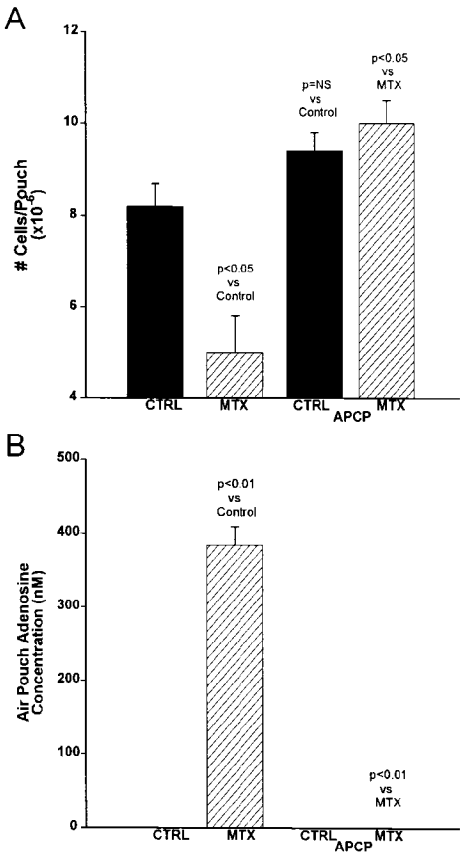

Figure 3. Injection of the ecto-5′-nucleotidase inhibitor APCP (100 μM) into an inflamed air pouch reverses the antiinflammatory effects of methotrexate and diminishes methotrexate-mediated increase in exudate adenosine concentrations. BALB/c mice were treated with four weekly intraperitoneal injections of saline (*CTRL*) or methotrexate (*MTX*, 0.5 mg/kg) before development of the air pouch and induction of inflammation by injection of carrageenan. In some animals, the carrageenan was suspended in saline containing the ecto-5′-nucleotidase inhibitor APCP (100 μM). Shown are the mean (±SEM) number of leukocytes accumulating in the inflamed air pouches (*A*) of six animals treated as described. Also shown is the mean (±SEM) exudate adenosine concentration of the exudates (*B*) from three of these six animals.

mined whether sulfasalazine, which appears to act by the same mechanism as methotrexate, also increases extracellular adenosine by a mechanism dependent on ecto-5′-nucleotidase–mediated dephosphorylation of AMP in the murine air pouch model. As with methotrexate, inhibition of ecto-5′-nucleotidase by APCP reversed completely the sulfasalazine-mediated increase in exudate adenosine ($P < 0.0007$, $n = 6$) and decrease in exudate leukocyte count ($P < 0.05$, $n = 6$; Fig. 4). These results are consistent with the previous demonstration that adenosine mediates the antiinflammatory effects of sulfasalazine in the murine air pouch model of inflammation, and confirm our previous demonstration that sulfasalazine and methotrexate share an antiinflammatory mechanism.

Discussion

We report here three lines of evidence supporting the hypothesis that ecto-5′-nucleotidase activity is required for methotrexate-mediated increases in extracellular adenosine. First, the relatively specific inhibitor of ecto-5′-nucleotidase, APCP, blocks completely the methotrexate-mediated increase in extracellular adenosine in supernates from HMEC-1 exposed to stimulated neutrophils. Second, cells that do not express ecto-5′-nucleotidase activity do not release adenosine under any conditions, but after transfection and expression of ecto-5′-nucleotidase on their surface, incubation with H_2O_2 after treatment with methotrexate does lead to adenosine release. Finally, injection of the ecto-5′-nucleotidase inhibitor APCP into the air pouch with the inflammatory stimulus prevents completely the methotrexate- and sulfasalazine-mediated release of adenosine into the inflammatory exudate, and reverses the antiinflammatory effects of methotrexate and sulfasalazine. These findings also exclude intracellular adenosine

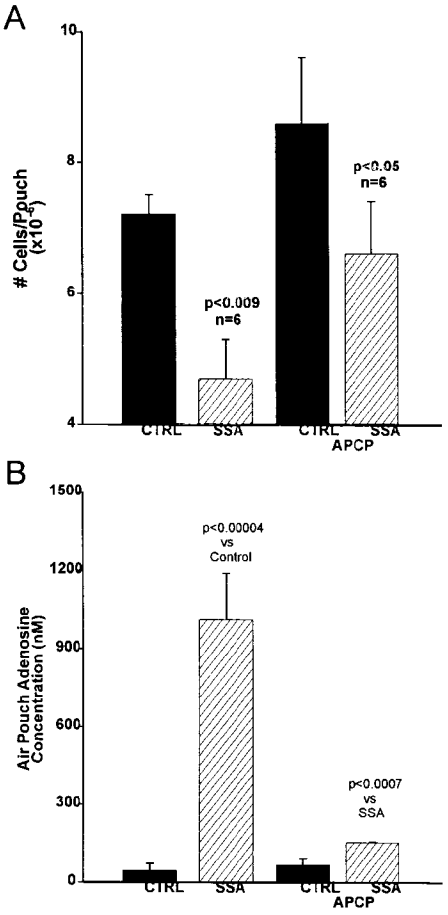

Figure 4. Injection of the ecto-5′-nucleotidase inhibitor APCP (100 μM) into an inflamed air pouch reverses the antiinflammatory effects of sulfasalazine and diminishes sulfasalazine-mediated increases in exudate adenosine concentrations. BALB/c mice were treated with saline (*CTRL*) or sulfasalazine (*SSA*, 100 mg/kg) by oral gavage for 3 d during the development of the air pouch. Inflammation was induced on the third day by injection of carrageenan, as described. In some animals, the carrageenan was suspended in saline containing the ecto-5′-nucleotidase inhibitor APCP (100 μM). Shown are the mean (±SEM) number of leukocytes accumulating in the inflamed air pouches (*A*) of six animals treated as described. Also shown is the mean (±SEM) exudate adenosine concentration of the exudates from these six animals (*B*).

generated as a consequence of adenosine deaminase inhibition as a source for the enhanced adenosine concentrations in the supernates of methotrexate-treated cells and the inflammatory exudates of methotrexate-treated animals.

Previous studies have demonstrated that the low levels of adenosine found in the supernatant of a variety of different cell types are derived from adenine nucleotides, but no previous studies have demonstrated that pharmacologic agents may enhance this mechanism for increasing extracellular adenosine concentrations. Thus, Kitakaze and co-workers have reported that neutrophils release adenosine which is derived primarily from the ecto-5′-nucleotidase–mediated dephosphorylation of adenine nucleotides (19). Similarly, endothelial cells release adenine nucleotides, and inhibition of ecto-5′-nucleotidase increases adenine nucleotide and decreases adenosine concentrations in the supernatant of these cells (20). Both of these observations depend on the use of an enzyme inhibitor which may have other effects on adenosine and adenine nucleotide metabolism as well. Although our studies do not address the precise metabolic source of extracellular adenine nucleotide released from normal or stressed cells, our results clearly demonstrate that ecto-5′-nucleotidase–mediated conversion of adenine nucleotides is required for methotrexate and sulfasalazine to increase adenosine release.

One mechanism by which methotrexate and sulfasalazine could increase extracellular adenosine is by directly increasing adenine nucleotide release as a result of cellular injury or necrosis. We found no evidence to indicate that methotrexate, H_2O_2, or their combination were toxic to 293T cells (resting or transfected) or HMEC-1 cells at the concentrations studied (LDH release). It is more likely that cell necrosis could increase adenine nucleotide release in the inflamed murine air pouches, although greater necrosis and destruction of pouch structures are, in fact, observed in the pouches of control than methotrexate- or sulfasalazine-treated animals. Moreover, there is a clear inverse correlation between exudate adenosine concentration and the number of leukocytes present in the tissue or the exudate of the air pouches (2, 3).

A second mechanism by which methotrexate and sulfasalazine could promote adenine nucleotide release is by promoting exocytosis of adenine nucleotide–containing intracellular vesicles. Both platelets and neurons release adenine nucleotides as a result of stimulated exocytosis, although none of the cells studied here have been shown previously to contain or release intracellular granules containing adenine nucleotides.

Another more likely explanation for the methotrexate- and sulfasalazine-mediated increase in extracellular adenosine is that methotrexate and sulfasalazine modulate purine nucleotide metabolism, and thereby promote the release of adenine nucleotides from the cells studied. Prior studies have demonstrated that human neutrophils release adenosine without exocytosis (13, 19, 21), and the apparent quantity of adenosine released is proportional to the "energy charge" of the cells (21). Madara and colleagues have demonstrated subsequently that neutrophils directly release AMP (22), which may be converted to adenosine by the action of ecto-5′-nucleotidase expressed on gut epithelial cells (19). Since methotrexate and sulfasalazine increase adenosine release from HMEC-1 and 293T cells only in the presence of a noxious stimulus (stimulated neutrophils or H_2O_2), it is most likely that methotrexate-induced adenine nucleotide (and thus, adenosine) release is enhanced only under conditions in which the energy charge of the treated cells or tissues is lowered, as occurs in inflammation. Whatever the metabolic steps involved, the mechanism by which adenine nucleotide accumulates extracellularly is not known. Although it is most likely that adenine nucleotide diffuses or is transported across the plasma membrane, the process by which this occurs remains a matter of speculation.

It is also possible that the methotrexate- and sulfasalazine-mediated increase in extracellular adenosine is due to blockade of adenosine uptake or use in the presence of a constant rate of release of adenine nucleotides. Thus, Deussen et al. (20) have reported that macrovascular endothelial cells release adenine nucleotide at a constant rate, and that the adenine nucleotide is converted extracellularly to adenosine under resting conditions. Moreover, maneuvers which enhanced adenine nucleotide release from endothelial cells or diminished adenosine use by these cells increased extracellular adenosine concentrations; a greater effect on extracellular adenosine production was observed when adenosine use was inhibited (20). However, it is unlikely that methotrexate is acting as a direct inhibitor of adenosine uptake, since methotrexate treatment increases extracellular adenosine but decreases extracellular hypoxanthine and inosine (1), a finding inconsistent with the hypothesis that purine uptake is diminished, since inosine and adenosine share a transporter. In contrast, diminished adenosine use in the presence of constant adenine nucleotide release may lead to increased extracellular adenosine concentrations, an increase which is marked at inflamed sites (23). As we have demonstrated previously, AICAR accumulates intracellularly after both methotrexate and sulfasalazine treatment (2, 3), and AICAR undergoes a cycle of dephosphorylation and adenosine kinase–dependent rephosphorylation similar to AMP (24). Although increased AICAR dephosphorylation–rephosphorylation has not previously been associated with cellular stress, the increased intracellular AICAR concentration present after methotrexate or sulfasalazine treatment may lead to competition with adenosine for adenosine kinase–dependent phosphorylation, and thereby diminish adenosine use. Others have reported that adenosine kinase activity is diminished under conditions of hypoxia, a phenomenon which contributes to increased adenosine release (25). Thus, although the effect of inflammation (or H_2O_2) on adenosine kinase activity has not been tested, it is possible that diminished adenosine use by adenosine kinase in the presence of modest increases in cellular AICAR concentration leads to a marked increase in extracellular adenosine concentration.

Both methotrexate and sulfasalazine, commonly used and effective antiinflammatory agents, diminish inflammation by promoting an increase in extracellular adenosine concentration. Our results indicate that the capacity of these antiinflammatory agents to promote an increase in extracellular adenosine concentration is completely dependent on the extracellular conversion of adenine nucleotides to adenosine. These observations suggest further that other agents that promote release of adenine nucleotides may prove to be effective for the treatment of such inflammatory diseases as rheumatoid arthritis.

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REVERSAL OF THE ANTIINFLAMMATORY EFFECTS OF METHOTREXATE BY THE NONSELECTIVE ADENOSINE RECEPTOR ANTAGONISTS THEOPHYLLINE AND CAFFEINE

Evidence that the Antiinflammatory Effects of Methotrexate are Mediated Via Multiple Adenosine Receptors in Rat Adjuvant Arthritis

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Objective. **Weekly low-dose methotrexate (MTX) remains the mainstay of second-line therapy for rheumatoid arthritis (RA). We have previously reported that adenosine, acting at specific receptors on inflammatory cells, mediates the antiinflammatory effects of MTX in both in vitro and in vivo models of acute inflammation, but the mechanism by which MTX suppresses the chronic inflammation of arthritis remains controversial. The present study was undertaken to further investigate the means by which adenosine mediates the antiinflammatory effects of MTX.**

Methods. **The effects of 2 nonselective adenosine receptor antagonists, theophylline and caffeine, were examined, using the rat adjuvant arthritis model of RA. These agents were given alone and in conjunction with MTX, and arthritis severity was assessed clinically, radiologically, and histologically. Since rodent adenosine A_3 receptors are not blocked by theophylline, selective A_1, A_{2A}, and A_{2B} receptor antagonists were tested as well.**

Results. **Control animals developed severe arthritis, which was markedly attenuated by weekly treatment with MTX (0.75 mg/kg/week). Neither theophylline alone nor caffeine alone (each at 10 mg/kg/day) significantly affected the severity of the arthritis, but both agents markedly reversed the effect of MTX as measured by a severity index, hindpaw swelling, and hindpaw ankylosis. Radiographic and histologic analyses confirmed these observations. Neither A_1, A_{2A}, nor A_{2B} receptor antagonists affected the capacity of MTX to ameliorate inflammation in adjuvant arthritis.**

Conclusion. **These results provide strong evidence that adenosine mediates the antiinflammatory effects of MTX in this model of RA. Moreover, the findings suggest that abstinence from caffeine, a ubiquitous food additive and medication, may enhance the therapeutic effects of MTX in RA.**

Low-dose, intermittently administered methotrexate (MTX) is among the most widely used forms of therapy for inflammatory arthritis (particularly rheumatoid arthritis [RA]), psoriasis, and inflammatory bowel disease. MTX was introduced for the treatment of inflammatory diseases, with very little understanding of its mechanism of action. We, and subsequently others, have reported that the antiinflammatory actions of MTX are mediated by its capacity to increase extracellular adenosine concentrations (1–4). However, the studies reported to date have demonstrated that adenosine is responsible for the antiinflammatory actions of MTX only in acute inflammation; the mechanism of action of MTX in the treatment of chronic inflammation has not been fully explored.

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It has been known since the work of Sattin and Rall (5) that adenosine modulates cellular behavior by interacting with specific receptors on the cell surface. It was subsequently recognized, using pharmacologic methods, that there were 2 distinct adenosine receptor subtypes, (6,7) and, more recently, cloning techniques have revealed the existence of at least 4 subtypes (A_1, A_{2A}, A_{2B}, and A_3) (for review, see ref. 8). Most of the known antagonists at adenosine receptors are methylxanthines, as documented in receptor binding and other pharmacologic experiments (9,10), and it is now generally accepted that the pharmacologic effects of theophylline and caffeine, 2 methylxanthines that are commonly encountered in medications and in foods and beverages, are mediated by antagonism of adenosine at its receptors (11). Pharmacologic studies with the murine air pouch model of acute inflammatory disease demonstrate that MTX-mediated increases in exudate adenosine inhibit inflammation via interaction with an A_2 (probably A_{2A}) receptor. Other pharmacologic studies have indicated that adenosine may also act at A_1 or A_3 receptors to inhibit inflammation (12–16).

To better understand the mechanism of action of MTX in the treatment of RA, we investigated whether the nonselective adenosine receptor antagonists theophylline (an agent that nonselectively blocks A_1, A_{2A}, and A_{2B}, but not A_3, adenosine receptors in the rat [17]) and caffeine (which blocks all receptors [9,10]), or more selective adenosine receptor antagonists reverse the antiinflammatory actions of MTX in the adjuvant arthritis model. We found that MTX inhibited the development of adjuvant arthritis and that blockade of A_1, A_{2A}, and A_{2B} receptors, but not the individual receptors alone, reversed the antiinflammatory effects of MTX.

MATERIALS AND METHODS

Materials. Heat-killed *Mycobacterium butyricum* was purchased from Difco (Detroit, MI), and Freund's complete adjuvant (CFA) was mixed as a 1% (weight/volume) suspension of the heat-killed bacteria in heavy mineral oil (Sigma, St. Louis, MO). MTX was purchased from Immunex (San Juan, PR), and methylprednisolone was purchased from Upjohn (Kalamazoo, MI). Theophylline, enprofylline, and caffeine were obtained from Sigma. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was obtained from Research Biochemicals (Wayland, MA), and ZM241385 was from Tocris-Cookson (Ballwin, MO). All other reagents used were the highest quality that could be obtained.

Animals. Female 8–12-week-old Lewis rats (Charles River, Wilmington, MA) weighing 130–190 gm were studied. The rats were housed in the New York University (NYU) animal facility, fed regular rat chow, and given access to drinking water ad libitum.

Induction of adjuvant arthritis. Arthritis was induced on day 0, by injection of 0.1 ml of CFA into the base of the tail. Synovitis developed 7–10 days postimmunization in 100% of the rats that did not receive any other treatment (18–20).

Treatment regimens. Animals were treated with a single weekly intraperitoneal injection of MTX (0.75 mg/kg/week in 1 ml of phosphate buffered saline) or a similar volume of saline, starting on the day of the injection of CFA (day 0) and continuing for the full 4 weeks of the experiment. Adenosine receptor antagonists were mixed into the drinking water of groups of animals to achieve a dosage of 10 mg/kg/day (adjusted daily to account for the weight and water intake of the animals); this dosage was higher than those previously reported to achieve effective levels in rats (21–24). All of these treatments were reviewed and approved by the Institutional Animal Care and Use Committee of NYU Medical Center and carried out under the supervision of the facility veterinary staff.

In each experiment, groups of 4–6 animals were treated as described, and each drug or combination was tested on at least 2 separate occasions. The control and MTX-treated groups were pooled from all of the experiments performed and consisted of 30 rats and 20 rats, respectively.

Arthritis assessments. The progress of arthritis was monitored by determining the ankle joint width, global arthritis severity index for swelling and erythema in 60 joints (scored on a scale of 0–3, with 0 representing no change and 3 representing most severe changes; maximum score of 180), and percentage of animals developing ankle joint ankylosis (assessed by the ability to extend/flex the joint). All measurements were performed on day 0 and biweekly for the duration of the study. Body weight was measured on day 0 and then weekly (18–20).

At the end of day 28, the rats were killed by Co_2 administration and, in some experiments, total-body radiographs were obtained (anteroposterior and lateral views), using a General Electric portable x-ray machine with a 3-second exposure (60-cm film-to-source distance). Radiographic scoring (18–20) was done based on the degree of soft tissue swelling, extent of bone erosion/destruction, bone mineralization, and joint space narrowing at both ankle joints. Radiographs were scored on a scale of 0–3 (0 = normal, 3 = maximum joint destruction) for each limb, by an observer who was blinded to the treatment group. The radiographic joint index score was then determined; this score represents the mean of the scores for both hind limbs from each rat, with a maximum possible score of 3 per rat.

Histopathologic analysis. Immediately after radiography, the hind limbs were removed just distal to the knee and placed in 10% buffered formalin. The fixed tissues were then decalcified and slides of sagittal slices through the hindpaw, stained with hematoxylin and eosin, were prepared using standard techniques. Slides were reviewed for soft tissue swelling, bone demineralization, pannus formation, cartilage erosions, and joint space narrowing.

Statistical analysis. The data were analyzed by analysis of variance, followed by analysis of differences between groups using Tukey's highest significant difference test performed with SigmaStat software (SPSS, Chicago, IL). All values are

reported as the mean ± SEM with the exception of ankylosis, which is reported as a simple percentage of the number of ankles.

RESULTS

Arthritis developed between 7 days and 10 days after injection and, similar to the findings in prior studies, disease in the control animals was characterized by increasing activity until day 20 and persistent joint inflammation through at least day 28 (at which time the experiment was terminated). The animals that were not injected with CFA did not develop arthritis (Figure 1). MTX treatment markedly attenuated the arthritis ($P < 0.00001$) (Figure 1A). Treatment with theophylline alone, a methylxanthine that blocks A_1, A_{2A}, and A_{2B}, but not A_3, adenosine receptors (17), appeared to diminish the activity of the arthritis at the early time points, although this difference did not reach statistical significance. More interestingly, theophylline markedly reversed the effect of MTX ($P < 0.003$) (Figure 1A). Injection of depot methylprednisolone completely abrogated the development of arthritis, and theophylline did not reverse the effect of methylprednisolone treatment (mean ± SEM severity index 0 ± 0 in rats treated with either methylprednisolone or methylprednisolone + theophylline, on any day of measurement).

As a separate indicator of arthritis activity, we measured hindpaw width. Injection of CFA caused a marked increase in the width of the hindpaw (Figures 1B and 2). Again, MTX significantly attenuated the hindpaw swelling in the CFA-treated rats ($P < 0.0001$). Theophylline did not diminish the increase in hindpaw width in these animals, but it partially reversed the antiinflammatory effect of MTX on CFA-induced hindpaw swelling, although this difference did not reach statistical significance. The greater severity of arthritis in the theophylline + MTX–treated rats, described above, appeared more marked than the difference in hindpaw swelling because it reflects, in addition to hindpaw swelling, involvement of a greater number of joints with more pain and ankylosis.

Ankylosis of the ankle joints was also assessed as an indicator of joint inflammation and destruction. Ankylosis was observed by the end of the study period in 94% of the ankles of the animals treated with either CFA alone or CFA + theophylline (Figure 1C). MTX diminished the percentage of animals that developed ankylosis, to 25%. The antiinflammatory effect of MTX was almost completely reversed by theophylline (63% of

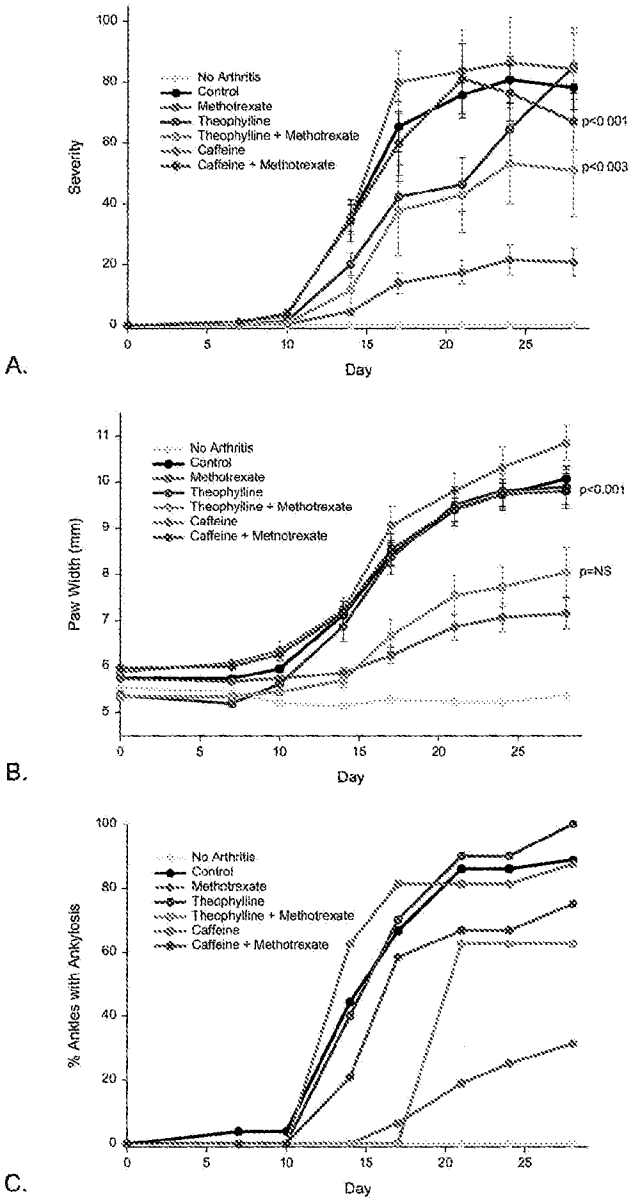

Figure 1. Effects of theophylline and caffeine on methotrexate (MTX) inhibition of the development of adjuvant arthritis. Rats were injected with Freund's complete adjuvant on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Severity index (**A**), hindpaw width (**B**), and ankylosis (**C**) were assessed twice weekly, as described in Materials and Methods. Treatment groups were as follows: 30 control rats, 20 MTX-treated rats, 8 theophylline-treated rats, 9 theophylline + MTX–treated rats, 12 caffeine-treated rats, and 12 caffeine + MTX-treated rats. In **A** and **B**, values are the mean ± SEM; in **C**, values are the mean. *P* values are versus the MTX-treated group. NS = not significant.

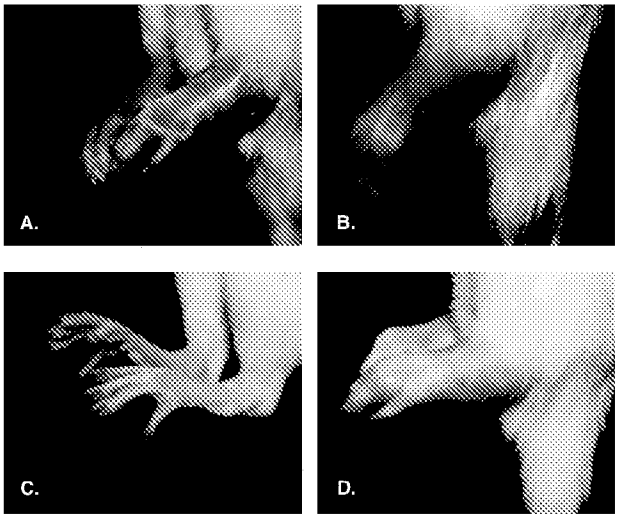

Figure 2. Effects of theophylline on methotrexate (MTX) inhibition of the development of adjuvant arthritis. Rats were injected with Freund's complete adjuvant (CFA) on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are the hindpaws of representative rats from each group, photographed on day 21. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline.

the animals treated with MTX alone developed joint ankylosis).

Analysis of joint radiographs at the termination of the experiment revealed changes consistent with those observed by physical examination. Both in the animals treated with CFA alone and in those treated with CFA + theophylline, there was complete destruction of the ankle joints (Table 1 and Figure 3). MTX treatment markedly diminished joint destruction. Again, coadministration of theophylline with MTX reversed the antiinflammatory effects of the latter compound ($P < 0.05$) (Table 1).

Table 1. Effects of theophylline on methotrexate (MTX)–mediated reduction of radiologic joint damage in adjuvant arthritis*

	Radiographic index, mean ± SEM	
	CFA	CFA + theophylline
No treatment	3.0 ± 0.0	2.7 ± 0.3
MTX, 0.75 mg/kg/week	0.3 ± 0.3†	1.6 ± 0.3‡

* Joint radiographs were obtained in 4 rats from each group and scored on a scale of 0–3 (0 = normal; 3 = complete destruction of the ankle joint), by an observer who was unaware of the treatment (see Materials and Methods). CFA = Freund's complete adjuvant.
† $P < 0.01$ versus no treatment.
‡ $P < 0.05$ versus MTX alone.

Histologic analysis (Figure 4) confirmed the clinical and radiologic findings. There was infiltration with inflammatory cells and almost complete loss of the normal joint architecture in the CFA-treated animals, and theophylline did not alter the histologic findings indicating joint destruction. MTX treatment preserved much of the joint architecture but theophylline completely reversed the effect of MTX, as reflected by the histologic changes.

To confirm that the effects of theophylline on MTX-mediated inhibition of inflammation were caused by adenosine receptor blockade in this model, we investigated whether another nonselective methylxanthine adenosine receptor antagonist, caffeine, also reversed the antiinflammatory effects of MTX. Like theophylline, caffeine alone did not significantly affect the onset or severity of arthritis in the rats (Figure 1A). Also like theophylline, caffeine reversed the antiinflammatory

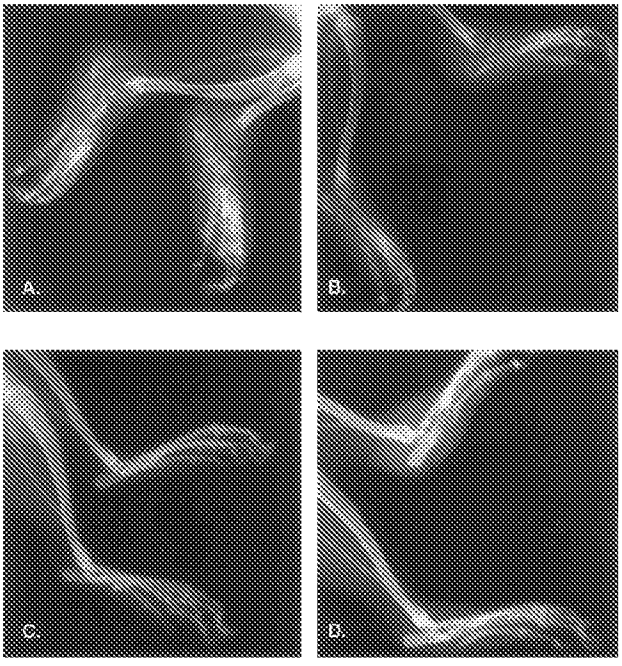

Figure 3. Effects of theophylline on MTX prevention of radiologic joint destruction in adjuvant arthritis. Rats were injected with CFA on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are representative radiographs obtained after the rats were killed on day 28. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline. See Figure 2 for definitions.

effects of MTX, whether measured as severity ($P < 0.001$), hindpaw width ($P < 0.001$), ankylosis, or radiologic changes ($P < 0.007$) (Figures 1A–C and Table 2). In contrast to either theophylline or caffeine, methylxanthines given in doses that have been administered to animals to selectively antagonize A_1 (DPCPX), A_{2A} (ZM241385), or A_{2B} (enprofylline [25]) receptors did not affect the capacity of MTX to diminish inflammation in this model (data not shown). Combinations of the more selective antagonists were toxic to the animals (causing cachexia, weight loss, and hair loss), and their effect on inflammation could not be evaluated.

During these experiments the animals continued to gain weight until they developed severe arthritis, at which point they began to lose weight, although none of the animals lost more than 20% of their pretreatment weight. Treatment with individual agents did not appear to have a direct effect on the rate of weight gain (26).

DISCUSSION

To date, the mechanism of action of MTX in the treatment of inflammatory arthritis has not been fully established (for review, see ref. 27). We report here that theophylline and caffeine, 2 chemically related, nonselective adenosine receptor antagonists, reverse the antiinflammatory effects of MTX in the adjuvant arthritis model of RA. This is the first direct demonstration in an in vivo model that adenosine mediates the antiinflammatory effects of MTX in chronic inflammatory arthritis. The results of the experiments reported here are consistent with the prior demonstration that adenosine mediates the antiinflammatory effects of MTX in acute inflammation both in vitro and in vivo (1–3). In contrast to prior reports, however, the present results indicate that adenosine must ligate multiple receptors in order to suppress chronic inflammation.

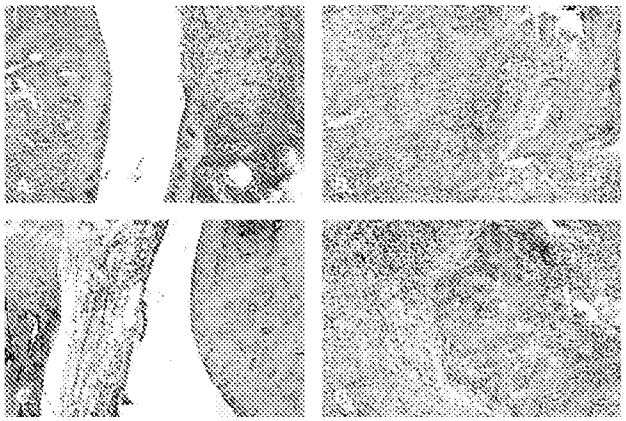

Figure 4. Effects of theophylline on MTX prevention of histologic features of joint destruction in adjuvant arthritis. Rats were injected with CFA on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are representative histologic sections obtained after the rats were killed on day 28. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline. See Figure 2 for definitions.

Table 2. Effects of caffeine on methotrexate (MTX)–mediated reduction of radiologic joint damage in adjuvant arthritis*

	Radiographic index, mean ± SEM	
	CFA	CFA + caffeine
No treatment	2.4 ± 0.2	2.0 ± 0.5
MTX, 0.75 mg/kg/week	0.7 ± 0.2†	2.1 ± 0.3‡

* Joint radiographs were obtained in 4 rats from each group and scored on a scale of 0–3 (0 = normal; 3 = normal to complete destruction of the ankle joint), by an observer who was unaware of the treatment (see Materials and Methods). CFA = Freund's complete adjuvant.
† $P < 0.001$ versus no treatment.
‡ $P < 0.007$ versus MTX alone.

Humans have ingested caffeine in tea, coffee, and chocolate since time immemorial, and theophylline has been used therapeutically for nearly half a century, although its mechanism of action remains in dispute. Currently, theophylline and caffeine are thought to exert their pharmacologic effects primarily by acting as adenosine receptor antagonists (11) or by inhibiting cellular phosphodiesterases (28,29).

Phosphodiesterase inhibition is thought to account for the effects of theophylline in the treatment of asthma, despite the fact that concentrations required to inhibit phosphodiesterase are much greater than those achieved therapeutically. Theophylline and other methylxanthine and non-methylxanthine phosphodiesterase inhibitors raise intracellular cAMP concentrations. Intracellular cAMP in elevated levels suppresses inflammatory cell function and inflammation (28,29), and it has been suggested that this underlies the antiinflammatory effects of theophylline (30–32). Indeed, the use of phosphodiesterase inhibitors (including non-methylxanthine phosphodiesterase inhibitors) has been advocated for the treatment of asthma (28), and phosphodiesterase inhibitors suppress the inflammation of adjuvant arthritis (33) as well. Neither theophylline nor caffeine prevented or augmented the development of adjuvant arthritis in rats that were not exposed to MTX. Moreover, the selective adenosine A_{2B} receptor antag-

onist enprofylline, which is also a methylxanthine and is a more potent inhibitor of phosphodiesterase than is theophylline (34), neither diminished arthritis alone nor affected the capacity of MTX to diminish inflammation in this model. Thus, it is unlikely that phosphodiesterase inhibition accounts for the capacity of theophylline and caffeine to alter the effect of MTX observed in the model of inflammatory arthritis reported here.

Unlike more recently developed methylxanthine derivatives, theophylline and caffeine are nonselective adenosine receptor antagonists (10). We found that neither theophylline nor caffeine alone significantly altered the course of adjuvant arthritis in MTX-treated rats, an observation that suggests that endogenous adenosine levels in the inflamed joints are insufficient to diminish inflammation in this model. None of the agents interfered with the capacity of methylprednisolone to suppress the development of adjuvant arthritis, indicating that the reversal of the antiinflammatory effects of MTX by theophylline and caffeine is specific and limited to MTX. In light of the previous demonstration that adenosine mediates the antiinflammatory effects of MTX in acute inflammation (2,3), the results reported here are most consistent with the hypothesis that the dominant pharmacologic effect of theophylline and caffeine in this model of RA results from adenosine receptor antagonism.

The antiinflammatory effects, as well as other physiologic and pharmacologic effects, of adenosine are clearly mediated via adenosine receptors, and all 4 adenosine receptors appear to act, when occupied, as antiinflammatory receptors. We and others have demonstrated that the inhibitory adenosine receptors on neutrophils, the inflammatory cells involved in acute inflammation, are A_2 (most likely A_{2A}) receptors (for review, see ref. 35). This finding has been confirmed by our subsequent demonstration that an adenosine A_2 receptor antagonist reverses the antiinflammatory effects of MTX, and by inference adenosine, in the murine air pouch model of inflammation (2). Adenosine A_1 receptor agonists have been reported to be the most potent antiinflammatory adenosine receptor agonists in other in vivo models of acute inflammation (12,36), although this finding may be accounted for by the effects of adenosine, via A_1 receptors, on the central nervous system (15).

MTX treatment has been shown to inhibit expression of collagenase by synoviocytes in biopsy specimens from patients with RA, and this specific inhibition of collagenase expression is most likely mediated by adenosine A_{2B} receptors (37,38). Several groups have reported that adenosine A_3 receptors, when occupied, diminish synthesis and release of cytokines, such as tumor necrosis factor α, that are thought to play a central role in the pathogenesis of RA (13–16,39). Because A_3 receptors in rodents are insensitive to theophylline (17), our results are most consistent with the surprising finding that blockade of A_3 adenosine receptors does not contribute to the antiinflammatory effects of MTX in this model of arthritis.

The biochemical mechanism by which MTX promotes adenosine release is not fully established. MTX is taken up by cells and polyglutamated; the polyglutamates of MTX remain metabolically active (40,41). It was originally suggested that MTX polyglutamates potently inhibit an intermediate enzyme in de novo purine biosynthesis, i.e., phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase (42,43), leading to intracellular accumulation of AICAR. Even the low doses of MTX used to treat inflammation in the mouse promote accumulation of AICAR in tissues (2), and recent studies confirm that long-term administration of MTX to rats with adjuvant arthritis promotes the accumulation of AICAR and its metabolites (44). Moreover, excretion of AICAR metabolites is increased in patients taking low-dose MTX for the treatment of psoriasis (45). The intracellular accumulation of AICAR has been associated with enhanced adenosine release (46), most likely as a result of AICAR-mediated inhibition of AMP deaminase (with extracellular accumulation of AMP). Indeed, the excess adenosine found in the supernates of MTX-treated cells or in the inflammatory exudates of MTX-treated mice is derived entirely from extracellular adenine nucleotides by the action of ecto–5′-nucleotidase, and the antiinflammatory effects of MTX in the murine air pouch model are completely blocked by ecto–5′-nucleotidase inhibitors (47). Alternatively, AICA-ribonucleoside inhibits adenosine deaminase, and this may also lead to adenosine accumulation (43,48–51). Whatever the mechanism, blood and urine adenosine concentrations are increased in patients who are taking MTX (45,52).

Although MTX is probably the most commonly used second-line agent for the treatment of RA, not all patients derive benefit from this drug, and treatment response is often less than complete (53). Caffeine is present in high concentrations in coffee, tea, chocolate, and soft drinks and as an ingredient of over-the-counter pain medications. The observation that caffeine completely reverses the antiinflammatory effects of MTX in this model of inflammatory arthritis suggests that avoidance of caffeine ingestion may enhance the efficacy of

MTX in the treatment of inflammatory diseases. However, before it can be recommended that patients taking MTX for the treatment of inflammatory arthritis avoid caffeine in their diets, further studies in humans should be undertaken.

The efficacy of low-dose MTX in the treatment of asthma is controversial (54–57). Our results suggest one explanation for inconsistency in the results of clinical trials of MTX in the treatment of this disease. Theophylline has long been used in the treatment of asthma, and theophylline usage by patients in these trials may have reversed or prevented any beneficial effects of MTX, thereby confounding the study results. Future studies of MTX for the treatment of asthma should control for theophylline use.

In conclusion, our findings further confirm the hypothesis that adenosine, generated endogenously, mediates the antiinflammatory effects of MTX, one of the most commonly used second-line drugs in the treatment of RA. These results indicate that other agents that promote adenosine release at sites of inflammation might also be useful for the treatment of RA and other forms of inflammatory arthritis and also suggest that avoidance of caffeine may enhance the efficacy of MTX in the treatment of inflammatory arthritis.

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Adenosine A_{2A} or A_3 Receptors Are Required for Inhibition of Inflammation by Methotrexate and Its Analog MX-68

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Objective. **Low-dose weekly methotrexate therapy remains a mainstay in the treatment of inflammatory arthritis. Results of previous studies demonstrated that adenosine, acting at one or more of its receptors, mediates the antiinflammatory effects of methotrexate in animal models of both acute and chronic inflammation. We therefore sought to establish which receptor(s) is involved in the modulation of acute inflammation by methotrexate and its nonpolyglutamated analog MX-68 (*N*-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]-carbonyl]-L-homoglutamic acid).**

Methods. **We studied the effects of low-dose methotrexate (0.75 mg/kg intraperitoneally [IP] every week for 5 weeks), MX-68 (2 mg/kg IP 2 days and 1 hour before induction of inflammation), dexamethasone (1.5 mg/kg IP 1 hour before induction of inflammation), or vehicle control on acute inflammation in an air-pouch model in A_{2A} and A_3 receptor knockout mice.**

Results. **Low-dose weekly methotrexate treatment increased the adenosine concentration in the exudates of all mice studied and reduced leukocyte and tumor necrosis factor α accumulation in the exudates of wild-type mice, but not in those of A_{2A} or A_3 receptor knockout mice. Dexamethasone, an agent that suppresses inflammation by a different mechanism, was equally effective at suppressing leukocyte accumulation in A_{2A} knockout, A_3 knockout, and wild-type mice, indicating that the lack of response was specific for methotrexate and MX-68.**

Conclusion. **These findings confirm that adenosine, acting at A_{2A} and A_3 receptors, is a potent regulator of inflammation. Moreover, these results provide strong evidence that adenosine, acting at either or both of these receptors, mediates the antiinflammatory effects of methotrexate and its analog MX-68.**

Low-dose weekly methotrexate is the "gold standard" of therapy in rheumatoid arthritis and other inflammatory diseases. Methotrexate's mechanism of action in the treatment of inflammatory diseases has been the subject of some controversy, although in previous studies, investigators in our group have demonstrated that adenosine mediates the antiinflammatory effects of methotrexate treatment in models of acute and chronic inflammation (1,2). Adenosine, whether released from injured cells or tissues or applied exogenously, regulates inflammation via interaction with one or more of the 4 known receptors for adenosine (A_1, A_{2A}, A_{2B}, and A_3), as demonstrated by many in vitro and in vivo pharmacologic studies (for review, see ref. 3). The demonstration that adenosine mediates the antiinflammatory effects of methotrexate in in vivo models of acute inflammation rests upon reversal of the antiinflammatory effects of methotrexate, either by enzymatic hydrolysis of adenosine by adenosine deaminase or by administration of adenosine receptor antagonists to reverse the antiinflammatory effects of methotrexate treatment (1,2). Although the antiinflammatory effects of methotrexate are mediated by multiple adenosine receptors in the adjuvant arthritis model of inflammation (2), the identity of the receptor(s) involved in the suppression of inflammation in models of acute inflammation has not been so well characterized.

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MX-68 (*N*-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]-carbonyl]-L-homoglutamic acid) is an analog of methotrexate chemically designed not to undergo intracellular polyglutamation (4). Like methotrexate, MX-68 has a high affinity for the enzyme dihydrofolate reductase and inhibits the proliferation of human peripheral blood mononuclear cells, endothelial cells, and synovial fibroblasts in vitro (5). In vivo, MX-68 prevents collagen-induced arthritis in mice (5) and rats (6), adjuvant-induced arthritis in rats (4), and autoimmune nephritis in lupus mice (7,8). Furthermore, both MX-68 and methotrexate increased the release of adenosine from Daudi cells, an effect that is mediated by inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, and inhibited the accumulation of leukocytes into the murine air pouch after carrageenan injection, an effect that was abolished by injection of the adenosine A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (9).

We investigated the pharmacologic mechanism by which methotrexate and its analog MX-68 diminish inflammation in the murine air-pouch model of acute inflammation. We report here that although both methotrexate and MX-68 suppress leukocyte and tumor necrosis factor α (TNFα) accumulation in the air-pouch model of acute inflammation in wild-type mice, neither suppresses inflammation in the air pouches of A_{2A} or A_3 receptor–deficient mice.

MATERIALS AND METHODS

Materials. Thioglycollate medium and carrageenan (type I) were obtained from Sigma (St. Louis, MO). Methotrexate was purchased from Immunex (San Juan, PR). MX-68 was a gift of Chugai Pharmaceutical (Tokyo, Japan). All other materials were of the highest quality that could be obtained.

Animal subjects. Mice with a targeted disruption of the gene for either the A_{2A} or A_3 adenosine receptors have been described in detail elsewhere (10,11). The mice used in these experiments were derived from 4 original heterozygous breeding pairs for each mouse. Mice described as wild type were specific for the related receptor knockout mice. Mice were housed in the New York University (NYU) animal facility, fed regular mouse chow, and given access to drinking water ad libitum. The experiments reported here were performed on male mice. All procedures described below were reviewed and approved by the Institutional Animal Care and Use Committee of NYU Medical Center and carried out under the supervision of the facility veterinary staff.

Polymerase chain reaction (PCR) confirmation of mouse genotype. DNA was extracted from the tips of mouse tails using a standard protocol. Briefly, tail tips were lysed in 500 μl of lysis buffer (100 m*M* NaCl, 20 m*M* Tris HCl [pH 8.0], 10 m*M* EDTA, 0.5% sodium dodecyl sulfate, 400 μg/ml proteinase K) overnight at 55°C. A saturated solution of NaCl (300 μl) was added to the lysed tips, and after 10 minutes on ice, tubes were centrifuged (16,000*g* at 4°C for 10 minutes). Genomic DNA present in the supernatant was precipitated by addition of 800 μl of isopropanol. Precipitates were washed once with 70% ethanol, vacuum dried, and resuspended in 30 μl of Tris–EDTA buffer.

The genomic DNA was then subjected to PCR using the following primers: 5′-AGCCAGGGGTTACATCTGTG-3′ (upstream) and 5′-TACAGACAGCCTCGACATGTG-3′ (downstream), which detect a 163-bp band for the wild-type A_{2A} allele; 5′-AGACAATCGGCTGCTCTGAT-3′ (upstream) and 5′-CAAGCTCTTCAGCAATATCACG-3′ (downstream), which detect a 618-bp band for the mutated A_{2A} allele; 5′-ACTTCTGGGCAGAAGTCTGACAAGA-3′ (upstream) and 5′-TTCGTCAACCCTGTTACCTGACTGT-3′ (downstream), which detect a 570-bp band for the wild-type A_3 allele; and 5′-ACTTCTGGGCAGAAGTCTGACAAGA-3′ (upstream) and 5′-AGATCTATAGATCTCTCGTGGGATC-3′ (downstream), which detect a 260-bp band for the mutated A_3 allele. To perform the PCR, 0.3 μg of genomic DNA was used in 30 μl of final reaction. The PCR was performed in a GeneAmp PCR System 2400 Thermal Cycler (Perkin-Elmer, Branchburg, NJ) under the following conditions: 95°C for 2 minutes, followed by 40 cycles of 94°C for 1 minute, 55°C for 20 seconds, and 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes.

Induction of air pouches and carrageenan-induced inflammation. To induce air pouches, 10–15-week-old male mice were injected subcutaneously on the back with 3 ml of air. After 2 days, the pouches were reinflated with 1.5 ml of air. On day 6, inflammation was induced by injection of 1 ml of a suspension of carrageenan (2% weight/volume in calcium- and magnesium-free phosphate buffered saline solution [PBS]) into the air pouch, as investigators in our group have previously described (1). After 4 hours, the mice were killed by CO_2 narcosis, the pouches were flushed with 2 ml of PBS, and exudates were harvested. Aliquots were diluted 1:1 with methylene blue (0.01% w/v in PBS), and cells were counted in a standard hemocytometer chamber (American Optical, Buffalo, NY).

Treatment with methotrexate, MX-68, dexamethasone, or vehicle. Animals were given weekly intraperitoneal (IP) injections of either methotrexate (0.75 mg/kg) or vehicle (0.9% saline) for 5 weeks, and experiments were carried out within 3 days of the last dose of methotrexate. MX-68 (2 mg/kg) was administered by IP injection 2 days and 1 hour prior to induction of inflammation in the air pouch, and dexamethasone (1.5 mg/kg) was administered IP 1 hour prior to induction of inflammation.

Quantitation of adenosine in inflammatory exudates. Aliquots of inflammatory exudates were added to an equal volume of 10% (w/v) trichloroacetic acid and kept on ice, followed by extraction of the organic phase with freon/trioctylamine (31/9). The aqueous phase was applied to a C-18 Sep-Pak cartridge (Waters, Milford, MA) and eluted off with methanol. After evaporation of the methanol, the samples were reconstituted in water, and the adenosine concentration was determined by reverse-phase high-performance liquid chromatography, as previously described (1). Samples were

applied to a μBondapack C-18 column (Waters, Milford, MA) and eluted with a linear 0–40% gradient of 0.01*M* ammonium phosphate (pH 5.5) and methanol formed over 70 minutes with a flow rate of 1.5 ml/minute. Adenosine was identified by retention time and by the characteristic ultraviolet absorption spectrum, and the concentration was calculated by comparison with standards, as previously described (1).

Quantitation of TNFα in air pouch exudates. After centrifugation (1,000*g* for 10 minutes), the cell-free exudates were collected. All exudates were kept frozen at −80°C until analyzed. The TNFα concentration was quantitated in the exudates in duplicate by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Statistical analysis. Overall differences among groups were analyzed by one-way analysis of variance. When overall analysis demonstrated the presence of significant differences among all of the different groups studied, the differences between specific groups were tested using a post-hoc analysis, Tukey's test. All statistical analyses were performed using SigmaStat software (SPSS, Chicago, IL).

RESULTS

Investigators in our group have previously demonstrated that adenosine, acting at A_2 receptors, mediates the antiinflammatory effects of methotrexate in a murine model of acute inflammation (1). Adenosine A_3 receptors have also been shown to mediate profound suppression of the inflammatory response (3). To further evaluate the role of adenosine and to better identify the adenosine receptor involved in methotrexate-mediated suppression of inflammation, we determined whether methotrexate inhibits leukocyte accumulation in the air-pouch model of acute inflammation in wild-type, adenosine A_{2A} receptor knockout, and adenosine A_3 receptor knockout mice.

In our initial experiments, we studied the inflammatory response in the A_{2A} and A_3 receptor knockout mice, and as we have previously reported (12), there were significantly fewer leukocytes in the air pouches of A_{2A} knockout mice than in the air pouches of their wild-type littermate controls (Table 1). The diminished accumulation of leukocytes in the air pouch exudates of A_{2A} knockout mice most likely results from the diminished number of blood vessels that form in the walls of the air pouches of the A_{2A} knockout mice (12). Interestingly, the adenosine concentration in the air pouch exudates of A_{2A} knockout mice was also significantly lower than that found in the wild-type controls (Table 2), possibly also a result of the diminished number of cells present. Both MX-68 and methotrexate increased the exudate adenosine concentration in the air pouches of wild-type, A_{2A} knockout, and A_3 knockout mice, although the increase in adenosine concentration in the air pouches did not achieve statistical significance for the exudates of the MX-68–treated A_{2A} knockout mice (Table 2).

As previously reported by investigators in our group and others (1,9), methotrexate and its analog MX-68 diminish air pouch leukocyte accumulation in response to carrageenan via enhanced adenosine release. Both agents reduced leukocyte accumulation in

Table 1. Leukocyte accumulation in inflammatory exudates*

Mouse group	Air pouch exudate ($\times 10^6$/ml)
A_{2A} knockout (n = 13)	2.02 ± 0.14†
A_{2A} wild type (n = 17)	2.73 ± 0.19
A_3 knockout (n = 18)	1.53 ± 0.09
A_3 wild type (n = 19)	1.93 ± 0.16

* Values are the mean ± SEM. Inflammatory exudates were induced in the air pouches of male knockout and control mice, as described in Materials and Methods. After 4 hours, the exudates were collected and the leukocytes were quantitated. The wild-type control mice were derived from the same heterozygous breeding pairs and matched for age.
† $P < 0.001$ versus A_{2A} wild-type mice, by one-way analysis of variance (Tukey's test).

Table 2. Adenosine concentration in air pouch exudates*

Treatment	Mouse group†		
	Wild-type	A_{2A} knockout	A_3 knockout
Control	76 ± 8	37 ± 4‡	63 ± 8
Methotrexate, 0.75 mg/kg	170 ± 28§	123 ± 23¶	129 ± 24#
MX-68, 2 mg/kg	142 ± 28**	71 ± 3	153 ± 28††

* Values are the mean ± SEM n*M*. Inflammatory exudates were induced in the air pouches of male knockout and control mice as described in Materials and Methods. After 4 hours, the exudates were collected and the adenosine was quantitated. Data for wild-type mice are a combination of those from both mouse strains. See Materials and Methods for dosing schedules.
† Of wild-type mice, 37 received saline control, 10 received methotrexate, and 9 received MX-68, an analog of methotrexate. Of A_{2A} knockout mice, 19 received saline control, 11 received methotrexate, and 7 received MX-68. Of A_3 knockout mice, 22 received saline control, 12 received methotrexate, and 7 received MX-68.
‡ $P < 0.004$ versus wild-type control mice, by one-way analysis of variance (ANOVA) (Tukey's test).
§ $P < 0.001$ versus wild-type control mice, by one-way ANOVA (Tukey's test).
¶ $P < 0.001$ versus A_{2A} knockout control mice, by one-way ANOVA (Tukey's test).
# $P < 0.01$ versus A_3 knockout control mice, by one-way ANOVA (Tukey's test).
** $P < 0.02$ versus wild-type control mice, by one-way ANOVA (Tukey's test).
†† $P < 0.004$ versus A_3 knockout control mice, by one-way ANOVA (Tukey's test).

the air pouches of wild-type mice (Figure 1), but neither agent inhibited inflammation in the air pouches of adenosine A_{2A} receptor–deficient mice. Surprisingly,

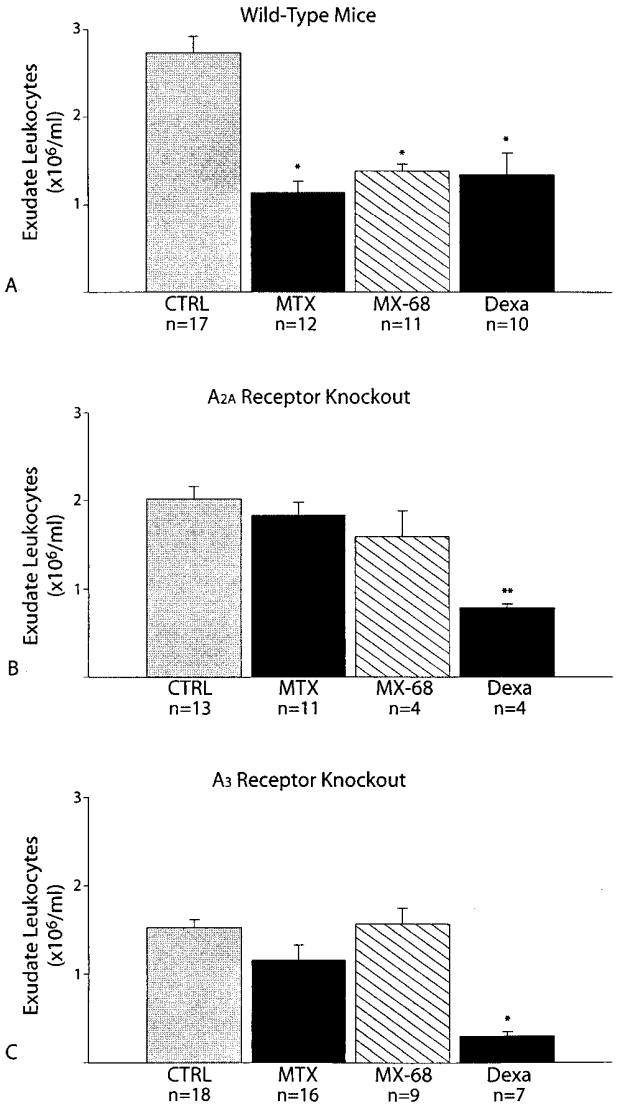

Figure 1. Effect of methotrexate (MTX), MX-68 (an analog of MTX), and dexamethasone (Dexa) treatment on leukocyte accumulation in air pouch exudates of A_{2A} and A_3 receptor knockout mice and wild-type controls. Male wild-type (**A**), A_{2A} receptor knockout (**B**), and A_3 receptor knockout (**C**) mice were treated with weekly injections of MTX (0.75 mg/kg) or saline control (CTRL) for 5 weeks prior to induction of inflammation, or they were treated with intraperitoneal injections of MX-68 (2 mg/kg) 2 days and 1 hour before induction of inflammation or with Dexa (1.5 mg/kg) or saline 1 hour before induction of inflammation. Inflammatory exudates were collected as described in Materials and Methods. Values are the mean and SEM. * = $P < 0.001$ and ** = $P < 0.05$ versus saline-treated mice, by one-way analysis of variance (Tukey's test).

Table 3. Tumor necrosis factor α (TNFα) concentration in air pouch exudates*

Treatment	Mouse group†		
	Wild-type	A_{2A} knockout	A_3 knockout
Control	377 ± 44	582 ± 81‡	255 ± 44
Methotrexate, 0.75 mg/kg	170 ± 27§	261 ± 51	140 ± 22
MX-68, 2 mg/kg	162 ± 29¶	464 ± 108	238 ± 63

* Values are the mean ± SEM pg/ml. Inflammatory exudates were induced in the air pouches of male knockout and control mice as described in Materials and Methods. After 4 hours, the exudates were collected and the TNFα was quantitated. Data for wild-type mice are a combination of those from both mouse strains. See Materials and Methods for dosing schedules.

† Of wild-type mice, 31 received saline control, 18 received methotrexate, and 10 received MX-68, an analog of methotrexate. Of A_{2A} knockout mice, 19 received saline control, 6 received methotrexate, and 10 received MX-68. Of A_3 knockout mice, 18 received saline control, 6 received methotrexate, and 12 received MX-68.

‡ $P < 0.03$ versus wild-type control mice, by one-way analysis of variance (ANOVA) (Tukey's test).

§ $P < 0.002$ versus wild-type control mice, by one-way ANOVA (Tukey's test).

¶ $P < 0.009$ versus wild-type control mice, by one-way ANOVA (Tukey's test).

neither methotrexate nor MX-68 significantly suppressed leukocyte accumulation in the air pouches of A_3 receptor knockout mice (Figure 1). Dexamethasone was equally effective at suppressing inflammation in the air pouches of wild-type, adenosine A_{2A} knockout, and adenosine A_3 knockout mice (Figure 1). Under the conditions studied, there was no difference in the type of white cells that accumulated in the air pouches of either treated or untreated wild-type or knockout mice (>90% polymorphonuclear leukocytes).

Similar to their effects on leukocyte accumulation, both methotrexate and MX-68 inhibited TNFα accumulation in the air pouches of wild-type animals (Table 3). Despite the diminished number of leukocytes in the inflammatory exudates of the A_{2A} knockout mice, there was a significantly higher concentration of TNFα in the air pouch exudates of these mice, most likely reflecting diminished endogenous suppression of inflammation by adenosine (13). In contrast, neither methotrexate nor MX-68 inhibited TNFα accumulation in the air pouch exudates of the A_{2A} or A_3 knockout mice (Table 3).

DISCUSSION

Low-dose weekly methotrexate treatment remains the standard against which all other new agents are compared in the treatment of rheumatoid arthritis

and other forms of inflammatory arthritis. Many different mechanisms of action for methotrexate have been proposed based on both in vitro and in vivo observations. The therapeutic effects of methotrexate have been ascribed to inhibition of lymphocyte proliferation or promotion of lymphocyte apoptosis (14–16) due to folate antagonism and diminished purine synthesis. However, the effect of methotrexate treatment on lymphocyte proliferation and apoptosis in patients is transient ($\leq$48 hours) and is reversed by folic acid. In contrast, the antiinflammatory effects of methotrexate are lasting and are not reversed by either folic acid or folinic acid (17–22).

Previous research in animal models has demonstrated that low-dose methotrexate treatment promotes intracellular accumulation of AICAR, an intermediate in purine synthesis, and that accumulation of AICAR is associated with increased adenosine release into inflammatory exudates (1,23); adenosine mediates the antiinflammatory effects of methotrexate treatment in animal models of both acute inflammation and adjuvant arthritis (1,2,24). In contrast, using adenosine receptor antagonists, Andersson and colleagues (25) did not confirm a role for adenosine in the antiinflammatory action of high-dose methotrexate (2–4 mg/kg/week; compared with 0.75 mg/kg/week in the present studies and in those reported in refs. 1 and 23, and compared with ~0.3 mg/kg/week in clinical practice) in the antigen-induced arthritis model in rats. Both the high doses of methotrexate and the uncertain pharmacology of the antagonists used could account for this difference, although it is also possible that different mechanisms of inhibition are involved in the suppression of this acute localized form of arthritis compared with adjuvant arthritis, a systemic form of arthritis.

Although the results of studies in animals do not always correlate with mechanisms of action in humans, previous studies have demonstrated that methotrexate treatment stimulates an even greater increase in adenosine release from cultured human fibroblasts and endothelial cells than that observed in the inflammatory exudates studied here (23). More important, oral low-dose methotrexate treatment leads to a marked increase in adenosine release from whole blood of patients and a significant increase of adenosine excretion into their urine (26,27). In contrast to the results reported in animals and in humans with rheumatoid arthritis and psoriasis, observations by Egan and coworkers (28) do not support the effect of methotrexate on adenosine release from inflamed bowel. In that study, however, patients were given a single subcutaneous dose of methotrexate and immediately underwent sigmoidoscopy. This experimental design permits neither redistribution of the drug to the intracellular compartment in affected tissues nor accumulation of intracellular AICAR required for enhanced adenosine release, a phenomenon which requires at least 48 hours in in vitro experiments (23) and weeks in animals treated with methotrexate (1).

Finally, ingestion of coffee, which contains the adenosine receptor antagonist caffeine, is associated with poor response to methotrexate therapy by patients with rheumatoid arthritis (29). Thus, the observations reported here and previously provide very strong evidence that adenosine mediates the antiinflammatory effects of low-dose weekly methotrexate therapy in a murine model of acute inflammation, and they support the hypothesis that adenosine mediates the antiinflammatory effects in patients as well.

Adenosine modulates cellular and organ function via occupancy of specific cell surface receptors, of which there are 4 known subtypes (A_1, A_{2A}, A_{2B}, and A_3). All are members of the large family of 7-transmembrane–spanning, heterotrimeric G protein–associated receptors (for review, see ref. 30). All 4 adenosine receptors regulate inflammation (for review, see ref. 31), although the A_{2A} receptor is considered to be the most important endogenous regulator of acute inflammation (13). In murine macrophages, A_{2A}, A_{2B}, and A_3 receptors have all been reported to diminish inflammatory cytokine secretion by macrophages (32–37), although in human macrophages, the A_{2A} receptor appears to be the dominant regulator of cytokine secretion (38,39). The differences in adenosine receptor predominance observed in these studies may have resulted from the different sources of macrophages studied (bone marrow, peripheral blood, peritoneum) or from altered adenosine receptor expression in monocyte/macrophages after release from the bone marrow (40) or following stimulation with various cytokines.

The results of the studies reported here demonstrate that in mice lacking either A_{2A} or A_3 receptors, methotrexate and MX-68 are not antiinflammatory, a finding consistent with the known antiinflammatory role of A_{2A} and A_3 receptors in acute inflammation (3,41). In studies of the air-pouch model of inflammation, investigators in our group had previously reported that A_2 receptors were primarily responsible for inhibition of inflammation (1), although the pharmacologic inhibitor used in that study apparently lacks specificity for A_3 receptors. In other studies, we have found that multiple adenosine receptors are involved in the antiinflammatory effects of methotrexate in adjuvant arthritis in the

rat, since only nonselective adenosine receptor antagonists (caffeine and theophylline), but not highly selective adenosine receptor antagonists, reversed the effects of methotrexate (2).

Adenosine is generated as a result of ATP catabolism, and is thus well suited for the role of metabolic regulator of such processes as coronary vasodilation in response to ischemia; adenosine concentrations increase from nanomolar to micromolar during ischemia as a result of ATP utilization (42–46). However, ischemia and increased work may not be the only stimuli for adenosine release. Other types of cellular injuries can lead to release into the extracellular space of adenosine or adenine nucleotides that can be converted extracellularly to adenosine (24,47–49). Moreover, necrosis of cells may occur following mechanical or inflammatory injury leading to release of intracellular contents, and ATP is present intracellularly in millimolar concentrations. Adenine nucleotides are also released into the extracellular space by ischemic or injured cells and tissues, where they are ultimately converted to adenosine by the action of ecto-5′-nucleotidase (24,47–49). Following sublethal injury, loss of as little as 5% of cellular ATP to adenosine may lead to 10-fold increases in extracellular adenosine (50,51).

Pharmacologic agents may also increase extracellular adenosine; investigators in our group and others have found that treatment of animals with low-dose weekly methotrexate, sulfasalazine, salicylates, or adenosine kinase inhibitors (including FK506) leads to increased adenosine concentrations and diminished leukocyte accumulation in inflammatory exudates (1,52–55). Similarly, adenosine uptake inhibitors may also possess antiinflammatory effects (56) by virtue of their capacity to increase extracellular adenosine concentrations.

Both methotrexate and MX-68 increase exudate adenosine concentrations 2–4-fold in wild-type, A_{2A} knockout, and A_3 knockout mice. Interestingly, adenosine concentrations were significantly lower in untreated A_{2A} knockout mice than in wild-type mice, although the methotrexate-induced increment in adenosine concentration in the air pouches of the knockout mice was similar to that observed in wild-type controls. One explanation for the lower adenosine concentration in exudates from A_{2A} receptor–deficient mice is increased activity of cellular adenosine transporters. Krauss and colleagues (57) and Diamond and Gordon (58) have previously reported that occupancy of the adenosine A_{2A} receptor leads to cAMP-dependent inactivation of an adenosine transporter, resulting in further increases in extracellular adenosine. It is therefore likely that ambient adenosine levels in the exudates induce partial inactivation of the transporter in the wild-type mice, whereas the transporter is fully active in the A_{2A} receptor–deficient mice. Alternatively, fewer leukocytes were present in the inflammatory exudates of the A_{2A} knockout mice, and this could account for the diminished adenosine concentration in the exudates.

The physiologic and pharmacologic effects of adenosine, acting at one or another of its receptors, are observed in nearly every tissue and organ. The findings reported here confirm the antiinflammatory effects of adenosine acting at A_{2A} and A_3 receptors. Moreover, the results reported here provide strong evidence that adenosine mediates the antiinflammatory effects of methotrexate at doses relevant to those used to treat inflammatory arthritis. These results indicate that agents that interact with adenosine A_{2A} and/or A_3 receptors directly or promote adenosine release at sites of inflammation may be useful for the treatment of inflammatory conditions.

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Methotrexate in rheumatoid arthritis

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After half a century of use, methotrexate continues to be a cornerstone in the therapy of rheumatoid arthritis. Renewed interest in the 1980s has brought new insights into the mechanisms of action and safety of the drug. The use of combination therapy in rheumatoid arthritis has not masked the value of methotrexate in a competitive market in any way. We review the pharmacodynamics and pharmacokinetics as applicable to its clinical use as an anti-inflammatory and disease-modifying agent here.

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CONTENTS

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Introduction to the compound

Over 50 years have passed since the introduction of aminopterin (4-aminopteroyl-glutamic acid). Its success in achieving remission, albeit temporary, in children with acute lymphocytic leukemia heralded a new age in cancer chemotherapy. Shortly thereafter, the substitution of a methyl group for hydrogen at the 10 position gave rise to methotrexate (MTX) and the realization of its wide-ranging potential brought the application of this drug in the treatment of rheumatoid arthritis (RA) as early as 1951. To this day, it is still the gold standard of disease-modifying therapy in RA and, as such, it is the most widely prescribed disease-modifying antirheumatic drug (DMARD) for this disease in both North America and Europe [1], as reflected in the guidelines for managing RA issued by the American College of Rheumatology (ACR) [2], as well as in Latin America [3] and possibly elsewhere. Its popularity has not been overshadowed by the introduction of other DMARDs or, more recently, biological agents and, indeed, it forms the anchor among many combination regimens.

Chemistry

MTX is an analog of folate synthesized originally in the 1940s and designed to inhibit dihydrofolate reductase selectively [4,5]. The IUPAC name for MTX is (*S*)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)methylamino)benzamido)pentanedioic acid. The enzyme dihydrofolate reductase catalyzes the conversion of dihydrofolate to tetrahydrofolate, which is an active cofactor involved in the *de novo* synthetic pathways for purine and pyrimidine precursors of DNA and RNA required for cell proliferation. As shown in FIGURE 1, the structure of MTX contains *para*-aminobenzoic acid, glutamic acid and a fully oxidized pteridine ring that inactivates the molecule as a cofactor.

MTX enters cells either as the parent compound or after conversion in the liver to its metabolite, 7-hydroxy-MTX, via the reduced folate carrier [5,6]. Both compounds are converted intracellularly into a polyglutamate form by the enzyme folyl polyglutamate synthetase [5].

The polyglutamated form of MTX, which can have up to four new glutamic acid moieties, accumulates within cells and is retained for long periods [7]. The ability to inhibit the enzymes involved in the *de novo* purine and pyrimidine biosynthesis increases with the number of glutamate moieties [8]. Some of these actions are a result of the ability of MTX polyglutamates to inhibit the enzyme aminoimidazolecarboxamidoadenosineribonucleotide (AICAR) transformylase [9–11], which converts AICAR into formyl-AICAR [12].

Pharmacodynamic & pharmacokinetic profile

MTX is administered to RA patients either orally or parenterally. The usual dose given for the treatment of RA is in the region of 7.5–25 mg/week, although dosing is often dictated by tolerability.

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Oral bioavailability for low-dose MTX as used in RA is high. At doses of less than 15 mg/m^2, mean oral bioavailability is in the range of 60–70%, although wide variability exists among individuals. The uptake of MTX from the gastrointestinal tract is mediated by saturable transporters, principally reduced folate carrier (RFC)-1 [13]. A saturation effect exists at the higher doses as given to patients with malignancies and absorption is reduced but such high doses are never used in the treatment of RA [14–16]. Food consumption is not a major influence of MTX absorption at these low doses [17], although, in children, it has been suggested that oral bioavailability of MTX is highest when taken on an empty stomach [18].

Despite the long-acting nature of MTX as an anti-inflammatory agent in RA, MTX, as well as its metabolite 7-hydroxyMTX, are in fact short lived with a known serum half-life of less than 8 h for MTX and less than 12 h for 7-hydroxyMTX; MTX becomes undetectable in the serum after 24–52 h [5,19]. The resultant polyglutamates of MTX metabolism are the major active compounds responsible for the anti-inflammatory actions. Not only do MTX polyglutamate levels in erythrocytes serve as an indication of anti-inflammatory efficacy of the parent drug, tissue levels of the polyglutamates, which may remain detectable after a period of months, may sufficiently account for the long-lasting anti-inflammatory actions of MTX [19–21]. Elimination of MTX occurs mainly in the urine, although a small part is also excreted through the biliary tract. Nonsteroidal anti-inflammatory drugs (NSAIDs), used commonly in RA, may decrease glomerular filtration rate and, in theory, may reduce the rate at which MTX is eliminated, possibly resulting in increased toxicity. This, however, is thought to be of consequence only rarely in practice, although caution should be exercised [22]. Furthermore, alterations in MTX metabolism may occur with polymorphisms of genes coding for enzymes important in folate metabolism, such as methylenetetrahydrofolate reductase [23,24].

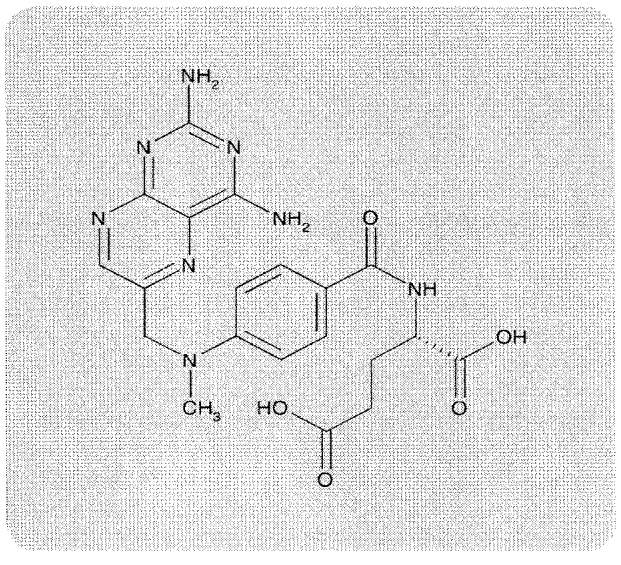

Figure 1. Chemical structure of methotrexate.

Clinical efficacy

The extensive experience of MTX has established this as a gold standard in the therapy of RA. Taken in once weekly doses in a wide range of 7.5–25 mg/week, it may be divided into three divided doses given 12 h apart or, more frequently, taken as a single dose all at once. Since a linear dose–response relationship has been found for many outcome variables, favoring a higher dose (10 mg/m^2) [25], it is recommended that the starting dose should be no lower than 10 mg/week, except in cases of low creatinine clearance [26–28]. While the oral route is by far the most common method of administration, it can also be given intramuscularly or subcutaneously, especially in cases of intolerance to oral administration. Its popularity took over 30 years to surface since its introduction in the early 1950s. While early reports in the 1980s re-established a place for MTX in RA, a number of randomized controlled trials in the mid-1980s brought wide acceptance for what is now an anchor drug in the treatment of RA. In a double-blinded crossover trial, Weinblatt and colleagues were able to demonstrate clinical improvement, including functional progress in 15 min-walking time and grip strength over a treatment period of 24 weeks [29]. These clinical benefits were echoed in a prospective, controlled, double-blind multicenter trial involving 189 patients [30]. The beneficial effects persisted during long-term therapy over 132 months [31]. Kremer and colleagues also demonstrated its safety and efficacy in a prospective study involving 29 patients with an average treatment duration of 29 months. Despite the small size of the population studied, there was clear evidence of radiographic regression of erosive disease as well as a reduction in prednisone requirement [32]. The protection against radiographic progression, however, has not been observed consistently and, indeed, has been questioned when MTX was compared with other DMARDs in this respect in a meta-analysis and benefit was only demonstrable against azathioprine [33]. Later studies reaffirmed the role of MTX as a radiological disease modifier, although the benefit may not be as great as was once thought [34].

More recently, with the growing interest in the newer generation of biological agents in the treatment of RA, it probably came as no surprise that MTX had to face new comparisons. While clinical improvement achieved with monotherapy with etanercept after 2 years may be greater than therapy with MTX, as determined by the ACR 20% criteria [35], differences were modest (72% for etanercept vs 59% for MTX) [36]. MTX, however, was clearly inferior to etanercept in protection against radiological deterioration, as has been confirmed in other studies. The same can be said of infliximab, which, when combined with MTX, resulted in better clinical response and quality of life. The combination of infliximab and MTX was well tolerated and was effective in suppressing radiological evidence of damage [37]. MTX has also been combined with rituximab in patients who

lacked a response to MTX alone. ACR 20, 50 and 70 responses were sustained at 48 weeks in patients treated with rituximab and MTX [38]. Similarly, concomitant MTX therapy was maintained in a study addressing the benefits of abatacept in RA [39]. The fully human monoclonal tumor necrosis factor (TNF)-α antibody, adalimumab, has also been used successfully in concert with MTX in RA patients with active disease despite treatment with MTX alone [40]. Clinical efficacy and safety of this regimen has been sustained over 4 years of evaluation [41].

In some sectors, a trend towards the early use of multiple agents for the treatment of RA has emerged over the last 10 years. O'Dell and colleagues first advocated the use of the combination of MTX, sulfasalazine and hydroxychloroquine in patients with poor responses to a single DMARD. After 2 years, patients on the 3-drug combination therapy had better clinical responses than MTX alone or a combination of sulfasalazine, and hydroxychloroquine, while the 3-drug combination did not result in more treatment withdrawals owing to drug toxicities [42]. These findings have been confirmed by others [43]. Boers and colleagues have used this approach in the aggressive early treatment of RA with high-dose prednisolone, MTX and sulfasalazine. While both MTX and prednisolone (starting dose 60 mg/day) were tapered then stopped in this 'step-down' regimen, the use of combination therapy was associated with a faster response to therapy, as indicated by a weighted change score of five disease activity measures (tender joint count, independent assessment on visual analog scale, grip strength, ESR and McMaster Toronto arthritis questionnaire). Radiological disease progression, as measured by the Sharp/Van der Heijde radiographic damage score, was also reduced by the use of combination therapy [44].

Safety & tolerability

In the mid 1980s, Kremer and colleagues demonstrated the efficacy of MTX in a small study [32]. The same study, however, raised concern over the frequency (90%) of toxicities associated with drug treatment and the regularity of elevation of transaminases (70%); all this despite a relatively low mean dose (12.4 mg/week) compared with what is used frequently today. Although the rate of toxicity was somewhat higher than observed in other studies, it was a concern that was nevertheless shared by others. Williams and colleagues, in a multicenter trial, found that withdrawal from MTX occurred in a third of those treated and, indeed, toxic effects are the main reasons for discontinuation of MTX therapy [30,45–47]. With evolving clinical practice, it is now clear that the long-term use of MTX rates among the safest of all antirheumatic treatments and elevation of transaminases leads to discontinuation of its use only rarely [1]. In fact, drug survival before discontinuation is higher for MTX than any of the other DMARDs used commonly for RA [48].

Gastrointestinal & hepatic toxicity

Nonspecific gastrointestinal side effects, including nausea, vomiting, dyspepsia, anorexia, stomatitis, aphthous ulcers and diarrhea, are very common (up to 40% [49]) and may often resolve on their own or respond to folic acid or dose reduction. In some cases of gastrointestinal intolerance, parenteral administration may alleviate symptoms.

The possibly most serious of side effects occur in the liver. The incidence of hepatotoxicity varies greatly and, while it was once believed to be extremely high, based on study populations of psoriatic patients treated with MTX [50], it would appear that the incidence is much lower in RA patients. There are many possible explanations for this, including higher doses used in psoriatic patients and effects of the disease itself. Walker and colleagues reported a 5-year cumulative incidence of serious hepatic side effects (cirrhosis or liver failure) of approximately 1 in 1000 patients, making this a very uncommon toxic effect encountered in the rheumatoid patient population. They further identified independent variables as predictors of serious liver toxicity, namely late age at commencing MTX and a long duration of therapy [51]. Based on the Roenigk histopathological grading system (I: mild steatosis, II: moderate steatosis, IIIa: mild fibrosis, IIIb: severe fibrosis, IV: cirrhosis), which was devised originally by dermatologists and used on psoriatic patients and which has since been criticized in some hepatology quarters for a lack of sensitivity [52,53], it has been suggested that serum aminotransferase levels are useful markers for predicting hepatic histology outcome, with abnormal prebiopsy mean ASTs linked strongly to abnormal biopsy grades [54]. Nevertheless, serum transaminase measurements are far from foolproof. Based on the accumulated information and taking into account the values as well as the dangers of interventions, such as surveillance through liver biopsies, the ACR has issued a set of guidelines for monitoring hepatic toxicity [55,56].

We have shown previously that release of the endogenous anti-inflammatory autocoid, adenosine, occurs in the liver during MTX therapy. The released adenosine is associated with both induction of collagen production as well as suppression of metalloproteinase synthesis, both of which contribute to the hepatic fibrosing effects of MTX *in vivo* [57]. The adenosine-mediated induction of collagen production in the liver is similar to that found in skin [58]. It is worth noting that ethanol also stimulates the release of adenosine by hepatocytes [57], which may provide a feasible mechanism for the induction of hepatic fibrosis by ethanol as well as the increased likelihood of development of cirrhosis in MTX-treated patients following ethanol consumption. In this respect, it is also worth noting that caffeine, a naturally occurring antagonist for adenosine receptors, is known to protect against the development of hepatic cirrhosis while, at the same time, it may interfere with the anti-inflammatory efficacy of MTX if consumed in large amounts (in excess of 180 mg/day) [59,60], although at least one study has suggested otherwise [61]. Other mechanisms of anti-inflammatory action have been proposed, including dihydrofolate reductase inhibition, reduced formation of the polyamines spermine and spermidine and alteration of the redox state in the cell. Other known risk factors for predisposition to MTX-induced hepatic injury include hepatitis B and C, diabetes, obesity and deficiency of α-1 antitrypsin.

Pulmonary involvement

The possibility of a diagnosis of MTX-associated pulmonary disease is often overlooked since the relatively nonspecific symptoms of cough, mild shortness of breath, fever or tachypnea are all too often ignored. The appearance on a chest radiograph of an interstitial infiltrate serves as an alert to the potential occurrence of a pneumonitis, which could indeed be fatal. Unlike MTX-induced hepatic fibrosis, the risk for which increases with cumulative dose, these pulmonary manifestations may occur very early on in the course of therapy, following only a few doses of MTX [62,63], suggesting that they are indeed the result of hypersensitivity phenomena. It is important to rule out infections and negative cultures are one of the three major criteria proposed for the diagnosis of MTX-induced lung injury [64,65]. The occurrence of opportunistic infections remains a real threat and a microbiological diagnosis may require the aid of bronchoalveolar lavage or a closed or open lung biopsy. Alarcon and colleagues identified previous use of DMARDs (especially sulfasalazine, gold, and D-penicillamine), hypoalbuminemia, old age, diabetes, smoking (particularly in men more than in women) and rheumatoid pleuropulmonary disease to be important predisposing factors for MTX-induced lung toxicity [65]. Treatment is empirical and may involve the use of high-dose corticosteroids and supportive therapy, including ventilatory support in severe cases, while discontinuation of MTX is essential. It is worth noting that recurrences may be fatal and occur in up to 50% of patients [62]. On the basis of this, it is recommended that MTX should not be reinstituted following the occurrence of this complication, although successful re-treatments have been documented in the literature [66].

In a Cochrane systematic review of double-blinded randomized placebo-controlled clinical trials, mucosal and gastrointestinal side effects of MTX were found to be reduced by co-administration of folic acid (5 mg/week) without altering activity [67,68]. Protection against the development of MTX-related side effects with folinic acid (5 mg/week or less) was not found to be statistically significant. Furthermore, these protective effects did not differ between either high or low dose folic acid or folinic acid [69]. The efficacy of MTX may, however, be reduced by folinic acid and the timing of folinic acid administration in relation to the MTX dose may be important in determining this effect [70,71] and it has been suggested that folic acid may also reduce the clinical efficacy of MTX [72].

Cytopenias of any one or combination of hematopoietic cell lines may occur during MTX therapy (<7% annually, even for MTX doses >15 mg/week [49]) and may be particularly serious in cases of existing renal failure. These are often mild and respond to dose reduction or folic acid supplementation. The possibility of coexistence of other folate deficiency states should be borne in mind. In severe cases, treatment with folinic acid (leucovorin) or respective colony-stimulating factors (e.g., granulocyte colony-stimulating factor) may be considered.

The possibility of an increase in the incidence of solid tumors has always been a concern with MTX treatment. However, evidence in support of this theory has not been consistent, particularly since RA is known to be associated with an increased incidence of lymphoma in any case [73,74]. In the light of reports of the regression of some of these tumors following discontinuation of MTX therapy, a direct causal relationship may well be justified [75–78]. However, the risks are extremely small and should not deter the clinician from the benefits of long-tested and efficacious drugs.

Conclusion

MTX remains the cornerstone of therapy for RA. The agent is nearly as effective in the therapy of RA as biologicals alone and the combination of MTX plus a biological agent is superior to biologicals or MTX alone. Because of its tolerability and efficacy, it is likely that MTX will remain in the therapeutic armamentarium for RA for years to come.

Expert commentary & five-year view

MTX is one of the most effective disease-modifying agents around for the treatment of RA. Over its long history, it has established itself as a gold standard in RA therapy. The many years of experience have also established a favorable toxicity profile, with

Key issues

- Methotrexate (MTX) is the most widely prescribed disease-modifying antirheumatic drug (DMARD) for rheumatoid arthritis (RA), despite the emergence of biologics.
- Polyglutamates are the major active compounds responsible for the anti-inflammatory actions of MTX.
- The release of adenosine constitutes one of the major mechanisms by which MTX exerts its anti-inflammatory effects and may account for the development of some of the treatment-related adverse effects, such as hepatic fibrosis.
- The oral route is the most common method of administration and the usual dose is in the region of 7.5–25 mg/week, although preferences vary and dosing is often dictated by tolerability. It may be taken as a single dose or in two or three divided doses.
- Overall differences in clinical efficacy are modest when MTX is compared with new biological agents or to other DMARD agents, although concomitant therapy improves clinical response.
- Caffeine consumption may reduce the anti-inflammatory efficacy of MTX in RA patients.

many of the previous anxieties and fears regarding malignant potential and hepatic toxicity now allayed in clinical practice. The emergence of newer biological agents has posed no threat to its existence; on the contrary, the fact that there is an improved clinical response when many of these biological agents are used in concert with MTX only strengthens its place in a competitive market. In fact, infliximab and rituximab are approved currently for use in RA only in combination with MTX. In addition, financial considerations important to treatment strategies in many parts of the world are in favor of MTX against the prohibitive costs of many of the newer biological agents. The arrival of other novel therapeutic modalities is unlikely to displace the safe stronghold MTX has in the market for RA, not only in the next 5 years but for many more years to come.

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Adenosine and Inflammation

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Abstract: Adenosine is a potent physiologic mediator that is released by cells following such stresses as hypoxia and exposure to reactive oxygen species (ROS). By binding to one or more of four known receptors, A_1, A_{2A}, A_{2B} and A_3 (all members of the family of G protein coupled receptors), adenosine suppresses inflammation and immunologic reactions. Here we review the expression and functional effects of these receptors on inflammatory cells and discuss the potential use of adenosine receptor agonists or agents that increase local adenosine concentrations in the treatment of inflammatory diseases or promotion of wound healing.

Key Words: Adenosine, adenosine receptors, P1 purinoceptors, methotrexate, wound healing, inflammation.

INTRODUCTION

Inflammation, the immune response to invading pathogens and the resolution of these responses culminating in wound healing are normal homeostatic responses to injury. Recent studies demonstrate that adenosine and its receptors help to link these responses and promote the smooth transition from acute inflammation to healing. The effects of adenosine receptor agonists and antagonists on inflammation, the immune response and wound healing will be reviewed here with attention to adenosine receptor-related developments in the treatment of inflammatory diseases and wound healing.

GENERATION OF ENDOGENOUS ADENOSINE

Following stress or hypoxia cells and tissues release adenine nucleotides which are converted extracellularly to adenosine by the actions of two enzymes, nucleoside triphosphate dephosphorylase (CD39) and ecto-5'nucleotidase (CD73)[1-10]. Adenosine is very short-lived in the extracellular space and once production decreases adenosine concentrations are restored to basal levels within seconds [7]. Extracellular adenosine mediates its physiologic and pharmacologic effects *via* interaction with one or more of four known classes of adenosine receptors (P_1 purinergic receptors), all of which are members of the large family of G protein coupled receptors (GPCR). Each of these receptors, designated A_1, A_{2A}, A_{2B} and A_3 [11], is capable of regulating immunologic and inflammatory reactions both *in vitro* and *in vivo*, as described below.

It is interesting to note that extracellular adenosine levels can reach remarkably high levels in the extracellular fluid surrounding malignant tumors and these dramatically elevated adenosine levels can suppress immunologic responses to tumors [12, 13].

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ADENOSINE RECEPTORS ON NEUTROPHILS

The potential anti-inflammatory effects of adenosine were first reported over 30 years ago, based on *in vitro* experiments. Adenosine was shown to inhibit the capacity of stimulated neutrophils to generate superoxide anion but not release of neutrophil granules following stimulation with the bacterial chemoattractant fMLP [14]. Although this work demonstrated that extracellular adenosine was responsible for the effects and that adenosine uptake was not required for adenosine to inhibit generation of reactive oxygen species it was not until 1985 that the effects of adenosine were shown to be mediated by interaction with a specific receptor [15-19] characterized at that time as an A_2 receptor. Once it became clear that adenosine A_2 receptors could be subdivided into A_{2A} and A_{2B} receptors it was shown that the effects of adenosine on stimulated neutrophil production of reactive oxygen species *via* interaction with specific A_{2A} receptors [20]. Studies of the effects of adenosine on other neutrophil functions *in vitro* revealed that occupancy of adenosine A_2 receptors (A_{2A} receptors judging from the pharmacology of the interaction) inhibited stimulated neutrophil adhesion to vascular endothelial cells and other surfaces [21-26]. Moreover, in inhibiting adhesion and generation of reactive oxygen species, adenosine receptor activation protects endothelial cells and other cells from injury by stimulated neutrophils [21-23, 27, 28]. In other experiments adenosine, acting at its receptors, inhibits neutrophil generation leukotriene B4, a potent lipid stimulus for neutrophils and other cell types [29]. Adenosine also inhibits stimulated neutrophil production of TNF *via* interaction with A_{2A} receptors as well [30].

Although adenosine A_{2A} receptor occupancy on neutrophils is responsible for the bulk of the adenosine receptor-mediated effects on neutrophil function these cells express other adenosine receptors which are capable of modulating neutrophil behavior. Interestingly adenosine A_1 receptors enhance some neutrophil functions inclu-ding neutrophil chemotaxis, adhesion to some surfaces and phagocytosis (and generation of reactive oxygen species) of immuno-

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globulin-coated particles *via* the Fc receptor [26, 31-33]. In other studies adenosine A_{2B} receptors inhibit neutrophil vascular endothelial growth factor secretion and thus limit vascular leak (edema formation, [34]).

Thus, *in vitro* studies of stimulated neutrophil function demonstrate that adenosine suppresses most of the inflammatory functions of neutrophils *via* A_{2A} receptors; in contrast A_{2B} receptors suppress vascular endothelial growth factor (VEGF) function by stimulated neutrophils and A_3 and A_{2B} receptors promote phagocytic function and increase the rate of chemotaxis, functions usually associated with inflammation [26, 32, 33, 35].

ADENOSINE RECEPTORS ON MACROPHAGES

Monocytes and macrophages express adenosine A_1, A_{2A}, A_{2B} and A_3 receptors under various conditions although there appear to be some species-related differences in the expression and function of adenosine receptors [36-45]. As with neutrophils adenosine A_{2A}, A_{2B} and A_3 receptors suppress inflammatory functions of monocytes and macrophages. Specifically, adenosine, acting at these receptors suppresses macrophage procoagulant production, TNFα, IL-1 and IL-6 production. In addition, adenosine inhibits superoxide generation and lysosomal enzyme secretion by stimulated monocyte/macrophages as well [36, 41, 46-48]. More recent studies have demonstrated that, in contrast to suppression of VEGF production by A_{2B} receptors, adenosine stimulates VEGF and IL-10 production by stimulated macrophages *via* interaction with A_{2A} receptors [49, 50]. By suppressing macrophage IL-12 production *via* A_{2A} and A_3 receptors adenosine also shifts adaptive immune responses as IL-12 pushes the immune response to a Th1-type response [50, 51]. Clearly the forgoing demonstrates the importance of adenosine receptors in regulation of innate immune and effector cells and, by altering the function of the cells of the innate response can influence the direction of the adaptive immune response.

DENDRITIC CELLS AND ADENOSINE RECEPTORS

Dendritic cells are thought to form the link between the innate and the adaptive immune systems. These cells take up antigens and process them and are the most potent antigen presenting cells for adaptive immune responses. Like nearly all other cells these cells also express adenosine receptors which clearly modulate their function. In one study adenosine receptor agonists diminished migration of dendritic cells *in vitro* and *in vivo* but did not affect other dendritic cell functions [52] however other studies indicate that adenosine A_1 receptor agonists increase migration and act as a chemotactic stimulus for dendritic cells [53, 54]. The adenosine A_{2A} receptor has been shown to regulate dendritic cell synthesis of chemokines and cytokines associated with Th1 immune responses such as TNF, IL-6, IL-12, interferon-α, CXCL10 and CCL17 [53, 54]. The effects of adenosine on dendritic cell function can also be regulated by endogenous adenosine deaminase which adheres to CD26, A_{2B} and A_1 receptors and, by eliminating endogenous adenosine at the cell surface, increases production of the Th1 cytokines interferon-γ, TNF and IL-6 [55].

IMMUNOLOGIC EFFECTS OF ADENOSINE RECEPTORS

The immunomodulatory effects of adenosine were first suspected over one quarter century ago soon after the demonstration that a significant proportion of children with Severe Combined Immunodeficiency (SCID) are genetically deficient in adenosine deaminase (ADA) activity [56], an enzyme that converts adenosine to inosine, a purine nucleoside which is not active at adenosine receptors, leading to accumulation of adenosine and deoxyadenosine [57, 58]. Although many of the immunosuppressive effects of ADA deficiency may result from adenosine accumulation it is also likely that lymphocyte depletion occurs as a result of accumulation of other more toxic metabolites (2'deoxyadenosine and 2'deoxyadenosine nucleotides). Nonetheless, these early studies sparked an interest in the effect of adenosine, adenosine receptors and adenosine receptor subtypes, which had only recently been described [59-61], on immune responses.

In 1978 Marone and colleagues first demonstrated the presence and function of an adenosine receptor on lymphocytes. Their initial finding, that adenosine A_2 receptors were present on lymphocytes, was based on the concentrations of adenosine required to stimulate a response and a characteristic increase in cAMP levels. Subsequent studies further supported the presence and function of adenosine A_2 receptors in the regulation of lymphocyte function [62]. Following from the cloning and differentiation of adenosine A_{2A} receptors from other receptors [63] and the development of selective agonists/antagonists for differentiating these receptors pharmacologically it has become clear that the primary receptor involved in regulation of lymphocyte function is the A_{2A} receptor. More recent studies have clearly demonstrated that the receptors primarily responsible for regulating lymphocyte function are A_{2A} receptors [64]. Moreover, although adenosine receptors may be present on B cells the primary site of adenosine receptor-mediated modulation of lymphocyte function is the T lymphocyte.

Initial studies of T cell function demonstrated that adenosine, acting *via* A_2 receptors suppressed lymphocyte function and that adenosine-mediated suppression of T lymphocyte function was defective in patients with Systemic Lupus Erythematosus [57, 65-68]. This finding has generally been supported by more recent studies with a deeper understanding of the mechanisms by which adenosine receptors suppress T lymphocyte function. As noted above, adenosine A_{2A} and A_3 receptors suppress IL-12 production by stimulated monocyte/macrophages leading to a Th1 type of immunologic response [50, 51]. Acting primarily at A_{2A} receptors adenosine downregulates immunologic responses by both suppressing proliferation and suppressing the production of the cytokines required to maintain a proliferative response [40, 69-76]. In addition, adenosine A_{2A} receptors suppress lymphocyte production of interferon-γ by stimulated T lymphocytes [77] further suppressing innate immune responses.

In addition to orchestrating the immunologic response lymphocytes may also play a direct role in response to infections and tumors and adenosine has been shown to suppress the function natural killer T cells via occupancy of both conventional and non-conventional adenosine receptors [78-82].

HUMORAL IMMUNE RESPONSES AND ADENOSINE RECEPTORS

The role of adenosine receptors in regulation of B cell responses to immunologic stimuli has not been established although these cells clearly do express adenosine receptors [83]. Nonetheless, adenosine, acting at its receptors on mast cells, may influence B cell function indirectly to stimulate production of IgE [84].

ADENOSINE RECEPTORS, INFLAMMATION AND TISSUE INJURY

Based on numerous *in vitro* studies, as described above, both endogenous adenosine and exogenous adenosine or adenosine receptor agonists would be expected to diminish inflammatory injury. In most cases adenosine A_2 (A_{2A} in more recent studies) receptors have been shown to be responsible for tissue protection and suppression of inflammation although A_1 receptor agonists are surprisingly anti-inflammatory in several models.

The earliest demonstrations that adenosine receptor occupancy could be used to protect tissue from injury were carried out in models of ischemia-reperfusion although transplant models and ureteral obstruction models have also been studied [25, 85-108]. In many of these models A_2 and A_{2A} receptor agonists were administered to animals following reperfusion or prior to injury although in other models it was shown that A_{2A} receptor knockout or antagonists potentiated inflammatory injury. Interestingly, A_1 adenosine receptors may also play a role in suppressing inflammation [35, 109-112] in several models. Although most of the anti-inflammatory effects of adenosine A_{2A} receptors are mediated *via* regulation of inflammatory cells at injured or inflamed sites the anti-inflammatory effects of adenosine A_1 receptors appear to stem from central nervous system effects [39, 113-137].

ADENOSINE-BASED ANTI-INFLAMMATORY DRUGS

Adenosine receptors are ubiquitous in their expression and there are many physiological and pharmacologic effects of adenosine acting at these receptors thus it was thought for many years that the development of selective adenosine receptor agonists for the treatment of inflammatory diseases might lead to a variety of unacceptable side effects in patients. Recent studies using highly selective A_{2A} receptor agonists have shown that, at appropriate doses, adenosine receptors on leukocytes can be selectively targeted without affecting blood pressure or other measurable physiologic functions [93, 101-103, 105, 118-123]. This finding is surprising and its applicability to humans remains to be critically tested, particularly for more chronic indications.

Early studies suggested that agents that increased local adenosine concentrations at inflamed sites might also be useful for the treatment of both acute and chronic inflammation. Thus, the discovery that adenosine mediates some if not all of the anti-inflammatory effects of methotrexate and sulfasalazine, two agents commonly used to treat Rheumatoid Arthritis and inflammatory bowel disease, was consistent with the utility and relative safety of this approach to suppressing inflammation [124-129]. Both methotrexate and sulfasalazine induce adenine nucleotide release from cells with conversion of nucleotides to adenosine taking place extracellularly [126]. The mechanism by which these agents appear to promote adenine nucleotide is unclear but both inhibit aminoimidazole-carboxamidoadenosine transformylase (AICAR transformylase) leading to accumulation of AICAR nucleotides intracellularly and increased AICAR nucleotide levels intracellularly leads to adenosine release [124-126, 128, 130, 131].

Other approaches to increasing extracellular adenosine concentrations to diminish inflammation have also been taken. By converting intracellular adenosine to AMP adenosine kinase is a critical regulator of extracellular adenosine levels. Selective inhibitors of adenosine kinase increase extracellular adenosine concentrations and suppress inflammation in several models [8, 110, 132-138]. Indeed, tacrolimus and cyclosporine also inhibit adenosine kinase and several studies have suggested that the tacrolimus- and cyclosporine-induced increases in extracellular adenosine contribute to the anti-inflammatory and immunosuppressive properties of these agents [139-141].

ADENOSINE AND WOUND HEALING

Inflammation is a critical response to tissue injury and noxious agents in the environment however most attention has been to controlling inflammation in those conditions characterized by an excessive or prolonged inflammatory response. Most inflammatory reactions resolve and tissue is repaired and, in some cases, regenerated. Thus, wound healing begins with the inflammatory response followed by the involvement of other cells and tissues. Recent studies have demonstrated that adenosine and its receptors are involved in the wound healing process and that topical application of adenosine receptor agonists can promote wound healing [142-145]. In most of these studies adenosine A_{2A} receptor agonists have been reported to promote wound healing [142, 144, 145] although in one study topical application of an A_1 receptor agonist promoted wound healing [143]. It is interesting to note that in the studies of A_{2A} agonists selective antagonists or knockout of the A_{2A} receptor blocked the effect of the topical A_{2A} agonists on wound healing whereas in the one study of A_1 receptor agonists high concentrations of the A_1 agonist were used so that selectivity may have been lost and no confirmation with receptor antagonists or knockout mice were performed.

In all of the studies of the effects of adenosine A_{2A} receptors in wound healing there were increased matrix and blood vessels (granulation tissue) in the agonist-treated wounds and, surprisingly, in the A_{2A} receptor knockout mice abnormal granulation tissue formation was noted in the wound bed [145]. Further investigations of the effect of adenosine A_{2A} receptors on wound healing demonstrated that adenosine A_{2A} receptors promoted formation of new blood vessels in the healing wound [145, 146].

Adenosine A_{2A} receptors promoted angiogenesis by three different mechanisms. Adenosine stimulates generation of known angiogenic mediators such as vascular endothelial growth factor (VEGF) by endothelial cells and macrophages [34, 49, 147-153] by both A_{2A} and A_{2B} receptors. It is likely that the different receptors reported to stimulate VEGF pro-

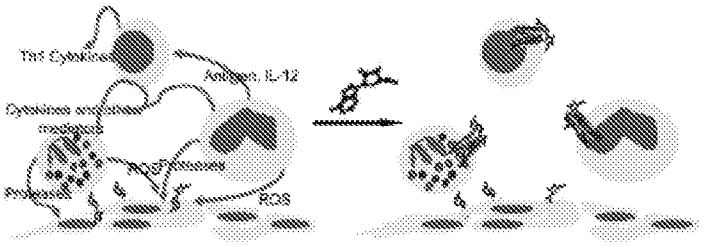

Fig. (1). Adenosine is an endogenous anti-inflammatory mediator.

duction and angiogenesis are important in different tissues although this is not clear. The second mechanism by which adenosine A_{2A} receptors promote angiogenesis is by inhibiting the production of anti-angiogenic factors such as thrombospondin I [154]. Finally, application of adenosine A_{2A} receptor agonists to wounds promotes the recruitment of bone marrow-derived endothelial precursor cells from the blood to the healing wound [146].

CONCLUSION

Adenosine and adenosine-based approaches to suppressing inflammation can form the basis for developing new anti-inflammatory drugs. Adenosine is released at sites of cellular and tissue injury and may act at one or more of its receptors to suppress the inflammatory functions of most cell types involved in both innate and adaptive immune and inflammatory responses. Approaches involving the development of selective receptor ligands as well as agents that promote adenosine release can provide new avenues for the development of anti-inflammatory agents.

Adenosine and adenosine receptors mediate the transition from inflammation to tissue repair. Testing of adenosine receptor agonists for the promotion of wound healing has already moved into the clinic. Other therapeutic applications of adenosine and its receptor-specific agonists may be in promoting angiogenesis at sites of tissue injury and repair. More speculative areas for development of adenosine-based angiogenic therapies include the treatment of malignancies. Rapidly growing tumors require angiogenesis and receptors for adenosine, which is found in high concentrations in the vicinity of tumors [12], may be useful targets for anti-angiogenic therapies.

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Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells

(inflammation/endothelium/fibroblast/adenosine receptor/purine receptor)

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ABSTRACT **Although commonly used to control a variety of inflammatory diseases, the mechanism of action of a low dose of methotrexate remains a mystery. Methotrexate accumulates intracellularly where it may interfere with purine metabolism. Therefore, we determined whether a 48-hr pretreatment with methotrexate affected adenosine release from [^{14}C]adenine-labeled human fibroblasts and umbilical vein endothelial cells. Methotrexate significantly increased adenosine release by fibroblasts from 4 ± 1% to 31 ± 6% of total purine released (EC_{50}, 1 nM) and by endothelial cells from 24 ± 4% to 42 ± 7%. Methotrexate-enhanced adenosine release from fibroblasts was further increased to 51 ± 4% (EC_{50}, 6 nM) and from endothelial cells was increased to 58 ± 5% of total purine released by exposure to stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils. The effect of methotrexate on adenosine release was not due to cytotoxicity since cells treated with maximal concentrations of methotrexate took up [^{14}C]adenine and released ^{14}C-labeled purine (a measure of cell injury) in a manner identical to control cells. Methotrexate treatment of fibroblasts dramatically inhibited adherence to fibroblasts by both unstimulated neutrophils (IC_{50}, 9 nM) and stimulated neutrophils (IC_{50}, 13 nM). Methotrexate treatment inhibited neutrophil adherence by enhancing adenosine release from fibroblasts since digestion of extracellular adenosine by added adenosine deaminase completely abrogated the effect of methotrexate on neutrophil adherence without, itself, affecting adherence. One hypothesis that explains the effect of methotrexate on adenosine release is that, by inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, methotrexate induces the accumulation of AICAR, the nucleoside precursor of which (5-aminoimidazole-4-carboxamide ribonucleoside referred to hereafter as acadesine) has previously been shown to cause adenosine release from ischemic cardiac tissue. We found that acadesine also promotes adenosine release from and inhibits neutrophil adherence to connective tissue cells. The observation that the antiinflammatory actions of methotrexate are due to the capacity of methotrexate to induce adenosine release may form the basis for the development of an additional class of antiinflammatory drugs.**

First reported to be useful in the treatment of rheumatoid arthritis (1), methotrexate is now widely used to treat a variety of inflammatory diseases, most notably rheumatoid arthritis (for review, see ref. 2). The mechanism by which methotrexate modulates inflammation remains, however, a mystery. The antineoplastic (antiproliferative) effects of methotrexate are due to inhibition of dihydrofolate reductase with resulting inhibition of purine and pyrimidine synthesis. However, folate depletion probably does not account for the therapeutic effects of methotrexate in inflammatory disease. (*i*) At the doses of methotrexate administered, leukopenia due to inhibition of DNA synthesis, does not occur (2), a finding not consistent with the hypothesis that methotrexate is antiinflammatory due to inhibition by methotrexate of dihydrofolate reductase. (*ii*) In two (3, 4) of three (5) published trials neither folate supplementation nor administration of reduced folate (folinic acid) reversed the therapeutic effects of this agent (although both agents reduced toxicity), direct evidence against inhibition of dihydrofolate reductase.

Recent observations have suggested a different mechanism to explain the antiinflammatory characteristics of methotrexate. Methotrexate and its polyglutamated analogues are very potent inhibitors of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (6–8), an enzyme required for *de novo* purine synthesis. In a study of canine myocardial injury Gruber *et al.* (9) found that administration of 5-aminoimidazole-4-carboxamide ribonucleoside (acadesine), the nucleoside precursor of AICAR, increases adenosine release from, diminishes neutrophil accumulation in, and increases collateral flow into ischemic myocardium. Thus, methotrexate, by inhibiting AICAR transformylase, may increase the intracellar concentration of its substrate, AICAR, which would lead, in turn, to increased release of adenosine, a potent antiinflammatory autocoid, at sites of inflammation.

We report that methotrexate, at pharmacologically relevant doses, induces adenosine release from human dermal fibroblasts and umbilical vein endothelial cells. The increase is most marked in the presence of neutrophils stimulated with the chemoattractant fMet-Leu-Phe (0.1 μM). In turn, the released adenosine inhibited neutrophil adhesion. Acadesine altered, in a manner similar to methotrexate, both adenosine release and neutrophil adherence.

MATERIALS AND METHODS

Materials. Tissue culture media [Dulbecco's modified Eagle's medium (DMEM) and medium 199] were obtained from GIBCO. [^{14}C]Adenine was purchased from NEN/DuPont and DEAE-cellulose thin layer chromatography plates were obtained from Eastman Kodak. The scintillant Filtron-X was supplied by National Diagnostics (Manville, NJ). Lymphoprep (Hypaque/Ficoll) was obtained from Nyegaard (Oslo). Trioctylamine was purchased from Aldrich Chemical (Orangeburg, NY) and Freon-113 was obtained from Matheson. Methotrexate, fMet-Leu-Phe, AICAR, and all other reagents were obtained from Sigma. All reagents were of the highest quality available.

Endothelial Cell Cultures. Endothelial cells were cultured and grown as described by Jaffe *et al.* (10). Briefly, segments of freshly obtained human umbilical veins were treated with

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ANOVA, analysis of variance; PMN, polymorphonuclear leukocyte.

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collagenase (0.1%), and the endothelial cells were collected and grown to confluence in gelatin-coated flasks containing medium 199/20% (vol/vol) fetal bovine serum at 37°C in a 5% CO_2/95% air atmosphere. The endothelial cells were then passed as necessary and grown to confluence in gelatin-coated 96-well tissue culture plates in medium 199/20% fetal bovine serum. All cells were used in the third passage.

Human Dermal Fibroblasts. Normal human fibroblasts (GM08389) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ) and cell line HM was a generous gift of Frank Martiniuk (New York Univ. Medical Center, New York). The cells were grown to confluence in DMEM/20% fetal bovine serum and passed as necessary. All cells were used during passages 5–15. Nearly identical results were obtained when either cell line was used.

Incubation of Cell Cultures with Methotrexate. Fibroblasts or preconfluent cultures of endothelial cells were washed three times with medium and then incubated for 48 hr at 37°C in a 5% CO_2/95% air atmosphere in fresh medium containing methotrexate at various concentrations. At the end of the incubation, cells were washed three times with fresh medium. When examined microscopically, there was no difference in cellular morphology between wells treated with methotrexate and those treated with medium alone.

Isolation of Neutrophils. Human neutrophils were isolated from whole blood after centrifugation through Hypaque/Ficoll gradients, sedimentation through dextran, 6% (wt/vol), and hypotonic lysis of erythrocytes. This procedure allowed study of populations that were 98 ± 2% neutrophils with few contaminating erythrocytes or platelets. Neutrophils were suspended in a Hepes-buffered saline solution consisting of 150 mM Na^+, 5 mM K^+, 1.3 mM Ca^{2+}, 1.2 mM Mg^{2+}, 155 mM Cl^-, and 10 mM Hepes (pH 7.45) (11).

Labeling of Connective Tissue Cells with [^{14}C]Adenine. After washing the cell cultures with fresh medium, cells were incubated in Hepes-buffered saline containing [^{14}C]adenine (25 μCi/ml; 1 Ci = 37 GBq) in a final volume of 250 μl per well for 3 hr at 37°C in a 5% CO_2/95% air atmosphere. At the end of this incubation wells were again washed three times with fresh medium before use in the experiments.

Assay for ^{14}C-Labeled Purine Release. To study the effects of preincubation with methotrexate or incubation with acadesine on ^{14}C-labeled purine release, endothelial cells or fibroblasts were incubated at 37°C in a 5% CO_2/95% air atmosphere in the presence or absence of 1.25×10^6 neutrophils per ml with or without fMet-Leu-Phe (0.1 μM) and acadesine in a final volume of 200 μl. This concentration of neutrophils is 12.5% of that which we have found (12) to injure endothelial cells. Because in preliminary experiments preincubation of connective tissue cells with acadesine markedly reduced [^{14}C]adenine uptake, acadesine was added during final incubations. In some experiments adenosine deaminase (0.125 international unit/ml), which had been dialyzed for 3–4 hr at 4°C against phosphate-buffered saline, was added to tissue culture wells. At the end of the incubation, samples of supernatant medium were collected, treated with 10% (vol/vol) trichloroacetic acid, and extracted with a mixture of Freon/trioctylamine, 31:9 (vol/vol), before centrifugation at 10,000 × *g*. The aqueous layer was then collected and frozen (−20°C) until assayed for purine content. In some experiments the remaining supernatant medium was removed, the remaining cells were lysed by overnight incubation with water, and the lysates were collected for quantitation of radioactivity. All experimental conditions were performed in duplicate with <5% variation between replicates. In preliminary experiments we found that addition of the chemoattractant fMet-Leu-Phe (0.1 μM) in the absence of neutrophils did not affect adenosine release from connective tissue cells regardless of whether or not they were treated with methotrexate (100 μM) or acadesine (100 μM, data not shown).

Separation and Quantitation of ^{14}C-Labeled Purines. A 50-μl portion of each sample was spotted onto DEAE-cellulose thin layer chromatography sheets. Separation was then carried out by chromatography in water/isobutanol/methanol/ammonium hydroxide in a ratio of 30:10:1:10 (vol/vol). After drying, the labeled purines and their carrier compounds (AMP, hypoxanthine, inosine, and adenosine, each at 500 mg/dl) were visualized under ultraviolet, cut out, and placed in scintillation vials. Radioactivity was quantitated in a Packard scintillation counter to an error of <0.2% (13).

Neutrophil Adherence to Endothelial Cell or Fibroblast Monolayers. After removal of medium for quantitation of purines, the monolayers and adherent neutrophils were fixed by addition of formaldehyde to 3.7% (vol/vol). Monolayers and their adherent neutrophils were then washed three times to remove nonadherent neutrophils and then stained with Weigert's hematoxylin. Adherent neutrophils were easily differentiated from underlying fibroblasts and endothelial cells on the basis of size and nuclear-staining characteristics (12). The number of neutrophils in three × 100 fields per well was quantified and the mean was calculated. Counts were performed on two replicate wells, which differed by <5%.

Statistical Analysis. All results represent the mean (± SEM), unless otherwise stated. The significance of the effects of agents and neutrophils and their interactions on adenosine release from and neutrophil adhesion to connective tissue cells was determined by the appropriate level of analysis of variance (ANOVA).

RESULTS

Treatment of fibroblasts with methotrexate caused a dose-dependent increase in release of adenosine from 4 ± 1% to a maximum of 31 ± 6% of the total purine released (Fig. 1). Methotrexate was a surprisingly potent promoter of adenosine release with an EC_{50} of 1 nM. When fibroblasts were treated with methotrexate and then incubated with neutrophils, there was a nearly identical dose-dependent increase in release of adenosine from 5 ± 2% to 23 ± 5% of total purine released (Fig. 1, $P < 0.01$). However, treatment of fibroblasts with methotrexate followed by incubation with neutrophils stimulated with fMet-Leu-Phe (0.1 μM) markedly enhanced

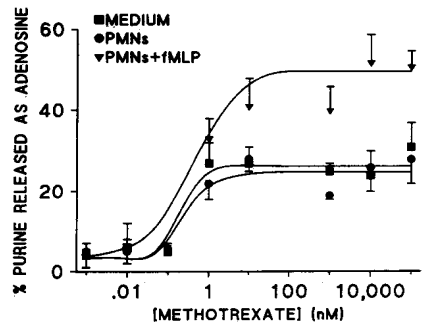

FIG. 1. Normal human fibroblasts were incubated with methotrexate at the indicated concentrations for 48 hr, washed, and labeled with [^{14}C]adenine. After washing, the fibroblasts were incubated in the presence of medium alone, neutrophils, or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils. After 2 hr the supernatant was collected and analyzed by thin layer chromatography. Data are the mean (± SEM) of four experiments performed in duplicate. Two-way ANOVA indicates that the percentage of purine released as adenosine varies with dose of methotrexate ($P < 0.0001$) and with the presence of stimulated neutrophils ($P < 0.003$). PMN, polymorphonuclear leukocyte; fMLP, fMet-Leu-Phe.

adenosine release from 4 ± 1% to 51 ± 4% of total purine released (Fig. 1). The quantity of adenosine detected corresponds to ≈400 nM, a concentration well within the effective physiologic range of activity for adenosine (14). Although stimulated neutrophils induced a greater shift in purine release from methotrexate-treated fibroblasts, there was no significant change in the concentration of methotrexate required to shift purine release (EC_{50}, 6 nM). As shown (Fig. 1), neither unstimulated nor stimulated neutrophils altered basal adenosine release from fibroblasts. Moreover, methotrexate or neutrophils or their combination did not affect total purine release from fibroblasts (7 ± 2, 7 ± 1, 7 ± 1%, respectively, of total purine pool released vs. 7 ± 1% from control cells).

As compared to fibroblasts, endothelial cells, under control conditions, released a greater percentage of their purine as adenosine (24 ± 4% vs. 4 ± 1%, $P < 0.01$, $n = 4$). When endothelial cells were treated with methotrexate (100 μM), there was an increase in the percentage of adenosine released to 42 ± 7% of total purine released ($P < 0.01$, $n = 4$). As with fibroblasts, unstimulated neutrophils did not affect the percentage of purine released as adenosine from control or methotrexate-treated endothelial cells (20 ± 4% and 39 ± 8%, respectively, $n = 4$). Stimulated neutrophils also did not affect adenosine release (18 ± 3% of total purine released, $n = 4$) from endothelial cells but increased adenosine release from methotrexate-treated cells to 58 ± 5% of total purine released ($P < 0.003$ vs. control, $n = 4$).

To examine the hypothesis that inhibition of AICAR transformylase by methotrexate is responsible for the release of adenosine, we determined whether acadesine, the nucleoside precursor of AICAR, also increases adenosine release. Acadesine (100 μM) induced fibroblasts to release a greater percentage of purine as adenosine (from 3 ± 1% to 19 ± 5% of total purine released, $P < 0.01$, $n = 4$; Fig. 2). However, acadesine was far less potent than methotrexate. As with methotrexate, stimulated but not unstimulated neutrophils also enhanced the effect of acadesine (100 μM) on adenosine release (53 ± 7% and 22 ± 13% of total purine released, respectively, $P < 0.001$, $n = 4$; Fig. 2).

Acadesine (100 μM) treatment increased the percentage of purine released as adenosine from endothelial cells from 24 ± 4% to 39 ± 6% ($n = 4$, $P < 0.01$). Stimulated neutrophils further increased the percentage of purine released as adenosine to 62 ± 9% of total purine released ($P < 0.001$, $n = 4$).

To determine whether methotrexate or acadesine was toxic to endothelial cells or fibroblasts, we compared both uptake and release of purine by treated cells. Cells treated with methotrexate (100 μM) took up as much [^{14}C]adenine as control cells (101 ± 7% of control uptake, $n = 4$) and did not release any greater percentage of the labeled purine pool during these experiments (7 ± 1 vs. 7 ± 2% of total label released from control and methotrexate-treated cells, $n = 4$). Similarly, fibroblasts treated with acadesine (100 μM) or exposed to stimulated neutrophils plus methotrexate (100 μM) also released no more of their labeled purine pool than control cells (7 ± 1 and 7 ± 1% of total label released). Moreover, no change in cell morphology was detected whether cells were treated with methotrexate, acadesine, stimulated neutrophils, or their combination. These results indicate that the increase in adenosine release from methotrexate-treated fibroblasts was not due to toxicity of methotrexate for fibroblasts. Similar results were obtained using endothelial cells (data not shown).

We next determined whether the release of adenosine from connective tissue cells treated with methotrexate was relevant to the antiinflammatory activity of methotrexate. We have previously demonstrated that adenosine, presumably acting at adenosine A_2 receptors on neutrophils, inhibits neutrophil adherence to endothelial cells (12). Therefore, we determined whether adherence by unstimulated and stimulated neutrophils to connective tissue cells was affected by treatment of the connective tissue cells with methotrexate. Treatment of connective tissue cells with methotrexate markedly inhibited adherence of both unstimulated and stimulated neutrophils to fibroblasts (EC_{50}, 9 nM and 13 nM, respectively, $P < 0.001$; Fig. 3). Similarly, acadesine also inhibited unstimulated and stimulated neutrophil adherence to fibroblasts (EC_{50}, 13 μM and 18 μM, respectively, $P < 0.001$; Fig. 4) at concentrations similar to those required for promotion of adenosine release. Methotrexate and acadesine inhibited neutrophil adherence to endothelial cells in a similar fashion (data not shown).

To determine whether the diminished adherence of neutrophils was related to the increase in adenosine release from connective tissue cells, we determined whether addition of adenosine deaminase, which metabolizes adenosine to inosine, reverses the effect of methotrexate treatment on neutrophil adherence. Adenosine deaminase alone did not affect adherence of either unstimulated or stimulated neutrophils to either fibroblasts or endothelial cells (Figs. 5 and 6). In contrast, and as described above, treatment of connective tissue cells with methotrexate (100 μM) markedly inhibited neutrophil adherence to connective tissue cells and this inhibition was completely abolished by the addition of adenosine deaminase. Similarly, adenosine deaminase completely reversed the effect of acadesine on adherence to endothelial

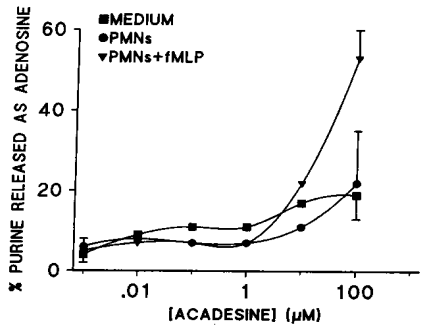

FIG. 2. After labeling with [^{14}C]adenine, normal human fibroblasts were incubated with acadesine at the indicated concentrations in the presence or absence of neutrophils or stimulated neutrophils (fMet-Leu-Phe at 0.1 μM). After 2 hr the supernatant was collected and analyzed by thin layer chromatography. Data are the mean (± SEM) of two to four experiments performed in duplicate. fMLP, fMet-Leu-Phe.

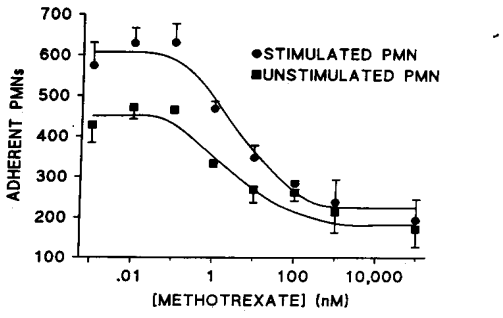

FIG. 3. Normal human fibroblasts were treated with methotrexate as indicated for 48 hr and then washed extensively. The fibroblasts were then incubated with neutrophils in the presence (stimulated) or absence of fMet-Leu-Phe (0.1 μM) for 2 hr. After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of four experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the dose of methotrexate ($P < 0.0001$) and with stimulation ($P < 0.0001$).

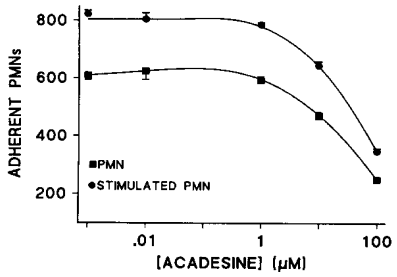

FIG. 4. Normal human fibroblasts were incubated in the presence of acadesine at the indicated concentrations with stimulated (fMet-Leu-Phe at 0.1 μM) or unstimulated neutrophils. After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of four experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the dose of acadesine ($P < 0.01$) and with stimulation ($P < 0.001$).

cells (Fig. 7). Nearly identical results were found with fibroblasts (data not shown).

DISCUSSION

The results of the experiments reported herein demonstrate an antiinflammatory action of methotrexate: increased adenosine release. Treatment of both fibroblasts and endothelial cells with methotrexate at pharmacologically relevant doses increases adenosine release from these cells, an effect that is even more marked in the presence of stimulated neutrophils. The adenosine released from methotrexate-treated connective tissue cells, in turn, inhibits adhesion of neutrophils to connective tissue cells, a critical initial step for infiltration or injury by neutrophils of connective tissue cells. These observations suggest that this is a mechanism by which methotrexate diminishes inflammation *in vivo*.

We have shown herein that the concentration of adenosine released from methotrexate-treated cells that remains extracellar (equivalent to a final concentration of 400–500 nM) inhibits neutrophil function but the antiinflammatory effects of extracellular adenosine are not confined to neutrophil function. Previous studies have demonstrated that adenosine occupies adenosine A_2 receptors on monocyte–macrophages (15–19) and lymphocytes (20–24), cells that play a major role in the pathogenesis of chronic inflammation. In general, occupancy of adenosine receptors on monocytes and lymphocytes inhibits their ability to induce tissue damage. It has been demonstrated (12, 14, 25–30) that adenosine occupies specific A_2 receptors on the surface of neutrophils to inhibit the generation of toxic oxygen metabolites such as O_2^-, H_2O_2, and adherence to endothelium. Thus, for example, increased release of adenosine from synovial cells could dampen both the acute and chronic inflammation present in the joints of patients with rheumatoid arthritis.

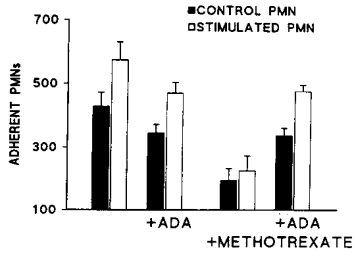

FIG. 5. Normal human fibroblasts were incubated with methotrexate (100 μM) for 48 hr, washed extensively, and then incubated for 2 hr with unstimulated or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils in the presence or absence of adenosine deaminase (ADA, 0.125 international unit/ml). After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of methotrexate ($P < 0.01$) and that adenosine deaminase induced a significant increase in adherence of both stimulated and unstimulated neutrophils to methotrexate-treated fibroblasts ($P < 0.05$).

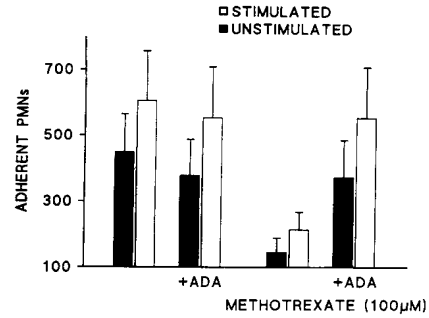

FIG. 6. Preconfluent monolayers of human umbilical vein endothelial cells were incubated in the presence or absence of methotrexate (100 μM) for 48 hr and washed extensively. The monolayers were then incubated with neutrophils in the presence (stimulated) and absence (unstimulated) of fMet-Leu-Phe (0.1 μM) and adenosine deaminase (ADA, 0.125 international unit/ml) for 2 hr. After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of methotrexate ($P < 0.01$) and that adenosine deaminase induced a significant change in adherence of both stimulated and unstimulated neutrophils to methotrexate-treated endothelial cells ($P < 0.05$).

Although the functional effects of adenosine are not restricted to a single type of inflammatory cell, we would predict that the effects of the adenosine released from methotrexate-treated cells would be restricted to the areas most directly infiltrated by inflammatory cells. Adenosine is very short-lived in whole blood where it is rapidly taken up by erythrocytes or metabolized by adenosine deaminase (31). Moreover, at sites of tissue necrosis intracellular enzymes such as adenosine deaminase are released that can metabolize adenosine to the functionally inactive purine riboside inosine.

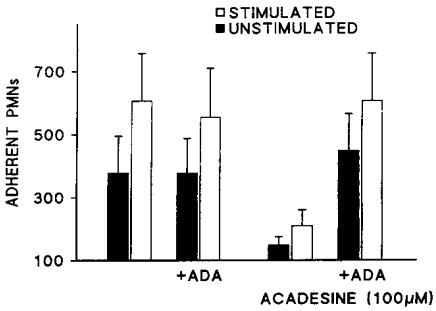

FIG. 7. Normal human endothelial cells were incubated for 2 hr with unstimulated or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils in the presence or absence of acadesine (100 μM) and adenosine deaminase (ADA, 0.125 international unit/ml). After fixation and washing, the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of acadesine ($P < 0.01$) and that adenosine deaminase induced a significant change in adherence of both stimulated and unstimulated neutrophils to acadesine-treated endothelial cells ($P < 0.03$).

The molecular mechanism by which methotrexate and acadesine promote adenosine release from connective tissue cells remains unknown; however, our data suggests one possible pathway by which methotrexate may induce increased extracellular adenosine concentrations. Methotrexate and its polyglutamated derivatives are potent inhibitors of AICAR transformylase (8). Inhibition of AICAR transformylase could cause accumulation of its substrate, AICAR and acadesine, a compound previously shown to promote adenosine release by an unknown mechanism (9). Our data are consistent with this hypothesis. Thus, the parallel effects of acadesine and methotrexate on adenosine release and neutrophil adherence suggest that the effect of methotrexate on adenosine release is due to inhibition by methotrexate of AICAR transformylase with accumulation of AICAR.

An alternative pathway by which methotrexate could modulate inflammatory cell interactions is suggested by studies of Nesher and Moore (32), who found that methionine reverses the effects of methotrexate on *in vitro* immunoglobulin production and hypothesized that uptake of methionine leads to regeneration of *S*-adenosylmethionine, a methyl donor that may be depleted in methotrexate-treated cells due to inhibition of dihydrofolate reductase. Our results do not exclude the hypothesis of Nesher and Moore (32) but suggest an alternative interpretation of their studies. In methotrexate-treated cells exogenous methionine may degrade to homocysteine that could recondense with adenosine thereby "trapping" excess adenosine intracellularly as *S*-adenosylhomocysteine. If increased adenosine release contributes to the antiinflammatory activity of methotrexate, then intracellular "trapping" of adenosine would reverse the effects of methotrexate treatment. Alternatively, methotrexate may inhibit the function of various cell types by different mechanisms.

Our results show that, in contrast to untreated connective tissue cells, cells treated with either methotrexate or acadesine release more adenosine after exposure to stimulated neutrophils. The mechanism by which stimulated neutrophils enhance adenosine release only from cells treated with methotrexate or acadesine is unknown. However, it is well known that intracellular stores of reduced gluthathione protect connective tissues from oxidant injury. Stimulated neutrophils release a variety of toxic oxygen metabolites that require detoxification and, ultimately, ATP turnover to regenerate reduced glutathione. Moreover, ATP is used to reestablish membrane ion gradients in connective tissue cells after exposure to the toxic products of neutrophils. Therefore, it is possible that neutrophils enhance adenosine release from connective tissue cells treated with methotrexate or acadesine because such treatment might diminish reutilization of adenosine generated during adenine nucleotide turnover.

Whereas our studies do not rule out a direct effect of methotrexate on neutrophil function, our results do indicate an antiinflammatory mechanism by which methotrexate may ameliorate rheumatoid arthritis; methotrexate increases adenosine release from connective tissue cells, specifically connective tissue cells under stress. Since the effects of adenosine are confined to those areas where the adenosine is released and because of the extremely rapid metabolism of adenosine in tissues and in the blood, the potential toxicity of excess adenosine release is reduced. Thus, the demonstration that agents capable of stimulating adenosine release at inflamed sites are antiinflammatory could lead to the development of an additional class of antiinflammatory drugs.

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The Antiinflammatory Mechanism of Methotrexate

Increased Adenosine Release at Inflamed Sites Diminishes Leukocyte Accumulation in an In Vivo Model of Inflammation

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Abstract

Methotrexate, a folate antagonist, is a potent antiinflammatory agent when used weekly in low concentrations. We examined the hypothesis that the antiphlogistic effects of methotrexate result from its capacity to promote intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) that, under conditions of cell injury, increases local adenosine release. We now present the first evidence to establish this mechanism of action in an in vivo model of inflammation, the murine air pouch model. Mice were injected intraperitoneally with either methotrexate or saline for 3–4 wk during induction of air pouches. Pharmacologically relevant doses of methotrexate increased splenocyte AICAR content, raised adenosine concentrations in exudates from carrageenan-inflamed air pouches, and markedly inhibited leukocyte accumulation in inflamed air pouches. The methotrexate-mediated reduction in leukocyte accumulation was partially reversed by injection of adenosine deaminase (ADA) into the air pouch, completely reversed by a specific adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), but not affected by an adenosine A_1 receptor antagonist, 8-cyclopentyl-dipropylxanthine. Neither ADA nor DMPX affected leukocyte accumulation in the inflamed pouches of animals treated with either saline or the potent antiinflammatory steroid dexamethasone. These results indicate that methotrexate is a nonsteroidal antiinflammatory agent, the antiphlogistic action of which is due to increased adenosine release at inflamed sites. (*J. Clin. Invest.* 1993. 92:2675–2682.) Key words: leukocyte • adenosine • purine • inflammation • methotrexate

Introduction

Methotrexate is a folate antagonist first developed for the treatment of malignancies and now widely used in the treatment of rheumatoid arthritis (1). Unlike its use in the treatment of malignancies (pulses of 20–250 mg/kg), methotrexate is administered weekly in low doses (0.1–0.3 mg/kg) to treat rheumatoid arthritis and other inflammatory diseases (1). Although the original rationale for the use of methotrexate in the treatment of rheumatoid arthritis was "immunosuppression," the molecular mechanism by which methotrexate suppresses inflammation is not well understood. It is unlikely that, in the doses given, methotrexate diminishes proliferation of immune cells by inhibiting de novo purine and pyrimidine synthesis since leukopenia and mucosal ulcerations, phenomena best explained by the antiproliferative effects of methotrexate, are considered evidence of drug toxicity and indications to decrease or stop therapy. Other proposed mechanisms include a decrease in neutrophil (but not macrophage) leukotriene synthesis (2) and inhibition of transmethylation reactions by inhibiting the formation of *S*-adenosyl-methionine, a methyl donor required for protein and lipid methylation (3).

We have recently proposed an alternative biochemical mechanism of action of methotrexate; methotrexate promotes the release of the antiinflammatory autocoid adenosine at inflamed sites (4). Previous studies have suggested that methotrexate accumulates within cells and, both directly and indirectly, inhibits 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)[1] transformylase, resulting in the intracellular accumulation of AICAR (Fig. 1; references 5–9). Increased intracellular concentrations of AICAR promote, by a complex mechanism, the increased release of the potent antiinflammatory autocoid adenosine (10, 11). Results of in vitro studies support this hypothesis (4); low concentrations ($\leq$ 10 nM) of methotrexate or higher concentrations of the cell-soluble, nonphosphorylated precursor of AICAR, AICARibonucleoside (acadesine), promote adenosine release from fibroblasts and endothelial cells. The increase in extracellular adenosine concentration diminished, via occupancy of specific cell surface receptors, the capacity of stimulated neutrophils to adhere to the methotrexate-treated endothelial cells and fibroblasts, in an in vitro model of an inflammatory interaction. Asako et al. (12) have confirmed that methotrexate suppresses inflammation by increasing adenosine release using the hamster cheek pouch model of acute inflammation but high concentrations of topically applied methotrexate (1 μM) were used in their study.

We report here the first evidence from in vivo studies that demonstrates that low-dose weekly methotrexate treatment causes intracellular accumulation of AICAR, increased adenosine release at sites of inflammation, and adenosine-dependent inhibition of inflammation. Moreover, we have confirmed that in methotrexate-treated mice adenosine diminishes inflammation via occupancy of adenosine A_2 receptors. These data provide the first in vivo demonstration of a novel biochemical mechanism of action for methotrexate.

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1. *Abbreviations used in this paper:* AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DMPX, 3,7-dimethyl-1-propargylxanthine.

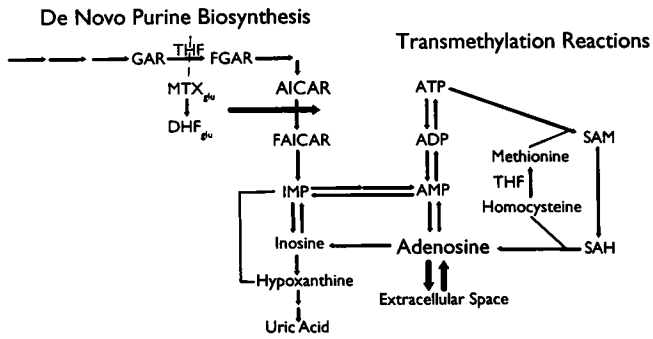

Figure 1. Proposed molecular mechanism of action of methotrexate. Shown here are the major steps in purine synthesis and degradation. Abbreviations: GAR, β-glycinamide ribonucleotide; FGAR, α-*N*-formylglycinamide ribonucleotide; MTX_{glu}, methotrexate polyglutamate; DHF_{glu}, dihydrofolate polyglutamate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, formyl-AICAR; IMP, inosinic acid; THF, tetrahydrofolate (reduced); SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

Methods

Materials. 3,7-Dimethyl-1-propargylxanthine and 8-cyclopentyl-dipropylxanthine were obtained from Res. Biochem. Inc. (Natick, MA). Injectable methotrexate (US Pharmacopeia) was purchased from Lederle Laboratories Division of American Cyanamid (Wayne, NJ). Adenosine deaminase (Type IV, calf intestinal), carrageenan, and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were the highest grade that could be obtained.

Induction of air pouches and carrageenan-induced inflammation. To induce an air pouch, mice (6-wk-old, female Balb/c mice, Taconic Farms Inc., Germantown, NY) were injected subcutaneously with 3 cc of air (on the back) three times per wk. After 3–4 wk, the air pouch was injected with 1 ml of a 2% (wt/vol) suspension of carrageenan and mice were returned to their cages, where they were allowed to run free for 4 h. At the end of 4 h, the animals were killed, 2 cm^3 of normal saline was injected into the pouch and the contents of the pouch were aspirated, diluted 1:2 with normal saline, and samples were taken for cell count. Smears of the undiluted fluid were prepared and an aliquot was stained (Wright-Giemsa), revealing that > 95% of the exudate cells were PMNs. In some experiments the air pouch was dissected from the subcutaneous tissue, fixed in formalin, processed for histologic sections, and stained (hematoxylin and eosin and Giemsa stains) using standard methods (13). These studies were reviewed and approved by the Institutional Animal Care and Use Committee of New York University Medical Center.

Treatment of mice with methotrexate. Mice were treated with weekly intraperitoneal injections (1 ml) of methotrexate (U.S.P., 0.05–0.5 mg/kg) or pyrogen-free (U.S.P.) normal saline (0.9%) for 3 or 4 wk. There were no apparent adverse effects of either treatment that could be detected by visual inspection of the animals and there were no apparent differences between the animals treated with saline and those treated with methotrexate.

Preparation of adenosine deaminase. Adenosine deaminase (50 μl, 4000 U/ml) was dialyzed against PBS overnight (4°C) before dilution and injection into the air pouch. For some experiments, dialyzed adenosine deaminase was incubated with deoxycoformycin (1 μM) for 30 min at room temperature before dilution (1:2600) in PBS containing carrageenan (14).

Injection of adenosine deaminase and adenosine receptor antagonists. In some experiments adenosine deaminase, previously inactivated adenosine deaminase (see above), and adenosine receptor antagonists were added to the carrageenan suspension to an appropriate final concentration. The final volume of the carrageenan suspension (with inhibitors) did not differ from that in control mice (1 ml).

Histologic analysis of sections of air pouches. Slides of stained (Giemsa) sections of mouse air pouch were examined microscopically using a Leitz research microscope to which was attached a high-resolution video camera. Video images were projected directly onto a screen for analysis by use of JAVA software (Jandel Sci., Corte Madera, CA) run on a Zenith 386 computer. All images were digitized directly and enhanced for contrast and brightness using PHOTOSTYLER software (Aldus, Inc., Seattle, WA).

Quantitation of AICAR. In some experiments the spleens were harvested and the cells were isolated by scraping through gauze. The cells were washed and resuspended at 100×10^6/ml in PBS. The cells were then lysed and the proteins were denatured by addition of 1 vol of trichloroacetic acid (10% vol/vol). The trichloroacetic acid was extracted with freon-octylamine and the supernatants were collected and stored at −80°C before analysis. Nucleotides were quantitated by HPLC by a modification of the method of Chen et al. (15). Briefly, nucleotides were injected onto a Partisil-10 SAX column (Whatman Inc., Clifton, NJ), isocratic elution with 0.007 M $NH_4H_2PO_4$, pH 4.0, followed by a linear gradient to 0.25 M $NH_4H_2PO_4$, pH 5, formed over 30 min at a flow rate of 2 ml/min. Absorbance was monitored at 250 and 260 nm and concentration was calculated by comparison to standards. Preliminary studies showed that 85% of added AICARibotide was recovered using this technique.

Adenosine determination. Aliquots of pouch exudates were added to a similar volume of trichloroacetic acid (10% vol/vol), followed by extraction of the organic acid, as described above. The adenosine concentration of the supernatants was determined by reverse-phase HPLC, as we have previously described (14). Briefly, samples were applied to a C18μBondapack column (Waters Chromatography Div., Milford, MA) and eluted with a 0–40%-linear gradient (formed over 60 min) of 0.01 M ammonium phosphate (pH 5.5) and methanol, with a 1.5 ml/min flow rate. Adenosine was identified by retention time and the characteristic UV ratio of absorbance at 250/260, and the concentration was calculated by comparison to standards. In some experiments the adenosine peak was digested by treatment with adenosine deaminase (0.15 IU/ml, 30 min at 37°C) to confirm that the peak so identified contained only adenosine (16). Preliminary studies demonstrated that 90% of added adenosine was recovered using this technique.

Digitization of chromatograms. Chromatograms were digitized using a Hewlett-Packard (Palo Alto, CA) Scanjet apparatus and the resulting images were enhanced for contrast and brightness using PHOTOSTYLER software run on a Zenith 386 personal computer.

Statistical analysis. The data were analyzed by the appropriate level of ANOVA performed by EXCEL 4.0 software (Microsoft, Inc., Bothell, WA).

Results

Low-dose weekly methotrexate markedly inhibits leukocyte accumulation in the air pouch in response to carrageenan. Low-dose weekly methotrexate is a potent form of antiinflammatory therapy in patients suffering from rheumatoid arthritis. To confirm that the murine air pouch model of inflammation was a reasonable model in which to study the antiinflammatory effects of methotrexate, we determined the effect of various doses of low-dose weekly methotrexate (administered intraperitoneally) on accumulation of leukocytes in the murine air pouch after injection of carrageenan. Methotrexate diminished, in a dose-dependent manner, the number of leukocytes that accumulated in carrageenan-treated air pouches by as much as 60% (IC_{50} = 0.08 mg/kg per wk, $P < 0.0002$ vs. saline-treated animals; Fig. 2). Moreover, the doses of methotrexate required to achieve a maximal antiinflammatory effect in this animal model are similar to those required for the treatment of rheumatoid arthritis (the equivalent of 10–15 mg/wk in a 70-kg individual). In other experiments we observed that metho-

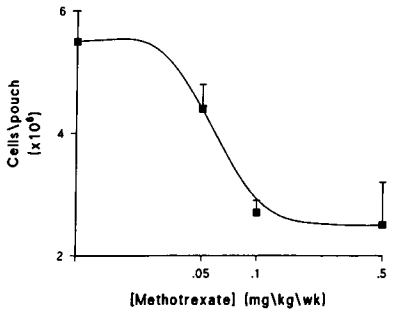

Figure 2. Weekly injection of low-dose methotrexate is antiinflammatory in the air pouch model. Methotrexate was given to the mice by intraperitoneal injection at the indicated doses for 3 to 4 wk during induction of the air pouch. The air pouch was injected with carrageenan (2% wt/vol), exudates were harvested 4 h later, and the cells were counted. Each point represents the mean (±SEM) of cell counts from three mice. Analysis of variance demonstrates that the exudate cell count varied significantly with the dose of methotrexate ($P < 0.0002$).

trexate-mediated inhibition of leukocyte accumulation was similar even when inflammation was induced up to 6 d after the last dose of methotrexate (data not shown).

Low-dose weekly methotrexate treatment increases intracellular concentrations of AICAR. We have proposed that the antiinflammatory actions of methotrexate result, both directly and indirectly, from the inhibition of AICAR transformylase (4). If this mechanism is correct, specific inhibition of AICAR transformylase should result in higher intracellular concentrations of AICAR. We directly tested the validity of this hypothesis by examining AICAR concentrations in splenocytes from saline- and methotrexate-treated mice (0.5 mg/kg by weekly intraperitoneal injection for 4 wk) by HPLC. We found that splenocytes from mice treated with methotrexate contained significantly more AICAR than those treated with saline (Table I, Fig. 3). These results are consistent with the hypothesis that low-dose methotrexate treatment leads to functional inhibition of AICAR transformylase.

Low-dose weekly methotrexate treatment increases adenosine concentrations in inflammatory exudates. We have previously shown that treatment of cells in culture with either methotrexate or AICARibonucleoside (acadesine), a nonphosphorylated, cell-soluble precursor of AICAR, promotes release of adenosine into the supernate and that adenosine release was

Table I. Methotrexate (0.5 mg/kg per wk) Treatment Increases Intracellular AICAR and Extracellular Adenosine

Condition	AICAR concentration (pmol/10^6 splenocytes±SEM)	Exudate adenosine concentration (μM, ±SEM)
	$n = 6$	$n = 16$
Control	26.5±10	0.57±0.09
Methotrexate (0.5 mg/kg per wk)	72.4±16*	1.11±0.19‡

Mice were treated with a weekly intraperitoneal injection of sterile saline or methotrexate for 4 wk during which time an air pouch was induced on the backs of these mice, as described. After 4 wk the air pouches were injected with carrageenan (2%wt/vol), the splenocyte lysates and inflammatory exudates were collected and analyzed by HPLC, as described. * $P < 0.02$ vs. control, Student's t test. ‡ $P < 0.008$ vs. control, Student's t test.

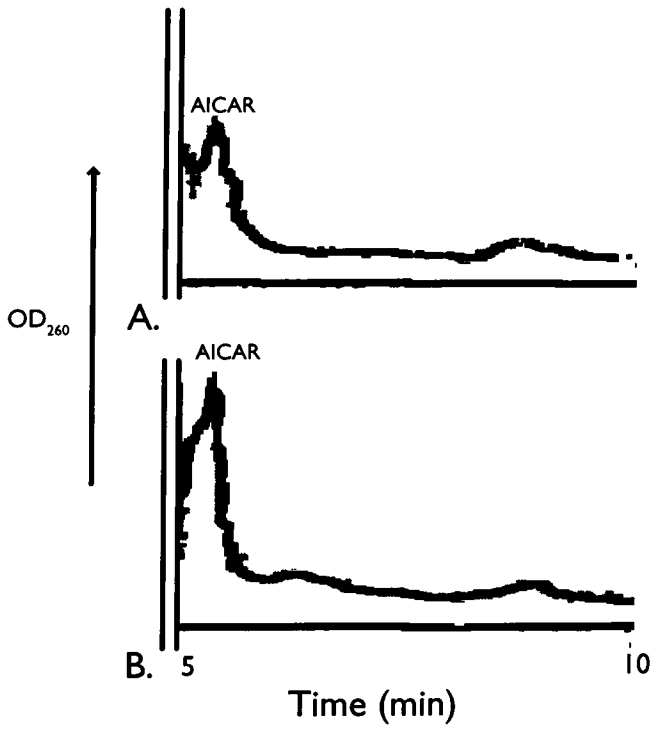

Figure 3. Intracellular concentration of AICAR is higher in splenocytes from animals treated with methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 4 wk during induction of the air pouch. After the animals were killed, and the air pouch exudate was harvested, the spleens were collected, and the cells were collected. The cell number was adjusted, the cells were lysed, and the AICAR-concentration analyzed by reverse-phase, ion exchange HPLC and detected at OD_{260}. Shown is a representative chromatogram of six of splenocyte AICAR from (*A*) a control (22.6 pmol/10^6 splenocytes) and (*B*) a methotrexate-treated mouse (87.3) pmol/10^6 splenocytes).

enhanced under conditions of "stress" (4). To determine whether low-dose weekly methotrexate treatment also promotes adenosine release in vivo we quantitated the adenosine concentration in inflammatory exudates taken from air pouches in saline- and methotrexate-treated (0.5 mg/kg per wk) mice. We found that methotrexate treatment led to a significantly higher adenosine concentration in the pouch exudate (Table I, Fig. 4). Thus, low-dose, intermittent methotrexate therapy promotes adenosine release at an inflamed site.

Adenosine mediates the antiinflammatory effect of methotrexate in the air pouch. To determine whether the methotrexate-induced increase in adenosine concentration observed in pouch fluid exudates was related to the antiinflammatory effects of methotrexate, we studied the effect of adenosine deaminase on leukocyte accumulation in methotrexate-treated mice. Adenosine deaminase irreversibly deaminates extracellular adenosine to its inactive metabolite, inosine, and thereby renders it inactive at adenosine receptors. Adenosine deaminase (0.15 IU/ml) did not significantly affect the number of leukocytes recovered from pouches of saline-treated animals, but partially reversed the antiinflammatory effect of methotrexate treatment (Fig. 5). Histologic examination of the air pouch tissue revealed that, similar to its effects on leukocyte counts in the exudate, methotrexate diminished leukocyte in-

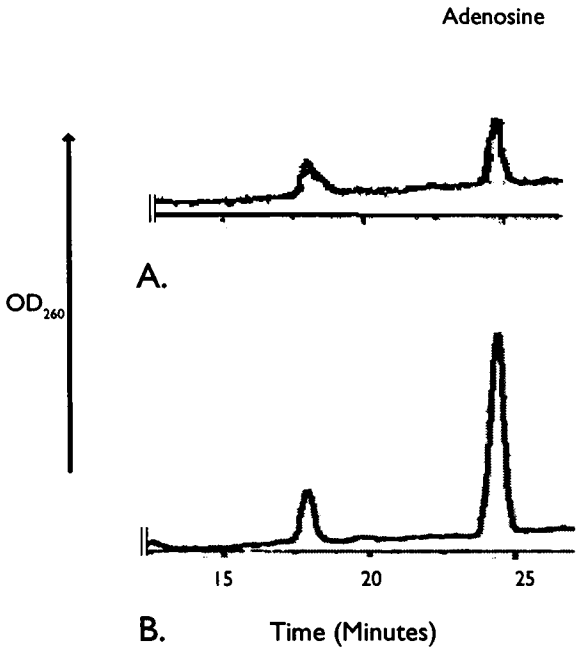

Figure 4. The concentration of adenosine is higher in exudates of mice treated with methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 4 wk during induction of the air pouch. After the animals were killed the air pouch exudate was harvested and soluble adenosine was extracted after treatment of the exudates with 10% trichloroacetic acid. The adenosine concentration of exudate extracts was then analyzed by reverse-phase HPLC, as described, and detected at OD_{260}. Shown is a representative chromatogram of 16 of pouch exudate adenosine from (*A*) a control (0.39 μM) and (*B*) a methotrexate-treated mouse (1.3 μM).

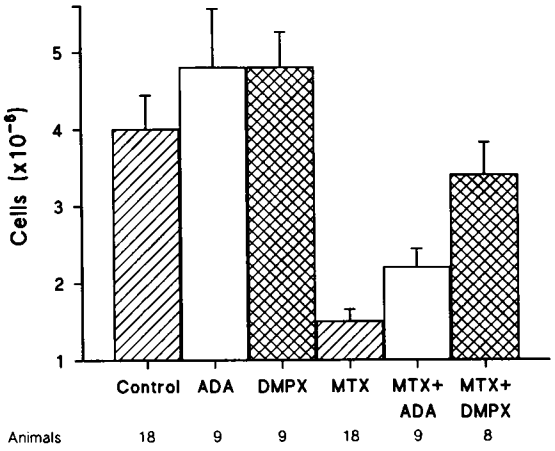

Figure 5. Adenosine deaminase (ADA, 0.15 IU/ml) and DMPX (mg/kg) reverse the antiinflammatory effects of methotrexate treatment (0.5 mg/kg per wk). Mice were treated with saline (control) or methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates. Methotrexate significantly inhibited the accumulation of leukocytes in the pouch exudate (4.0±0.4 vs. 1.5±0.1 $\times 10^6$ cells/pouch, control vs. methotrexate, $P < 3 \times 10^{-6}$). Neither ADA (4.8±0.5 $\times 10^6$ cells/pouch) nor DMPX (4.8±0.4 $\times 10^6$ cells/pouch) significantly affected the number of cells that accumulated in the control air pouches, but both ADA (2.3±0.8 $\times 10^6$ cells/pouch) and DMPX (3.8±0.5 $\times 10^6$ cells/pouch) significantly reversed the antiinflammatory effect of methotrexate ($P < 0.006$ and $P < 0.001$ vs. methotrexate alone, respectively).

filtration into the pouch tissue (38±2 vs. 106±14 cells/160× field, methotrexate vs. control, $P < 0.01$), and adenosine deaminase reversed the antiinflammatory effect of methotrexate (88±3 cells/160× field, $P < 0.01$ vs. methotrexate alone) without affecting leukocyte infiltration in control mice (91±6 cells/160× field, P = NS vs. control, Fig. 6). Adenosine deaminase-mediated reversal of the antiinflammatory effect of methotrexate treatment was specific since adenosine deaminase did not reverse the antiinflammatory effects of dexamethasone (1.5 mg/kg, injected intraperitoneally 1 h before injection of the pouch with carrageenan, Fig. 7). Moreover, conversion of adenosine to inactive metabolites was responsible for reversal of the antiinflammatory effect since adenosine deaminase which was inactivated by prior incubation with its tight-binding, irreversible inhibitor deoxycoformycin (1 μM), did not affect the antiinflammatory capacity of methotrexate treatment (data not shown). We conclude from these experiments that the increase in extracellular adenosine in the methotrexate-treated animals is responsible, at least in part, for the antiinflammatory effects of methotrexate.

The antiinflammatory effect of adenosine is mediated via adenosine A_2 receptors. There are at least two major subtypes of adenosine receptor, A_1 and A_2, that can be differentiated, in part, on the basis of agonist and antagonist specificity (17, 18). Since extracellular adenosine appeared to mediate the antiinflammatory effects of methotrexate, we sought to determine whether the antiinflammatory actions of adenosine were mediated by occupancy of a specific adenosine receptor. We therefore injected receptor-specific adenosine receptor antagonists into the air pouch with the inflammatory stimulus. The adenosine A_1 receptor antagonist 8-cyclopentyl-dipropylxanthine (0.2 mg/kg) did not affect leukocyte accumulation in the air pouch in either control animals or methotrexate-treated animals (Fig. 8). Because of its poor solubility in aqueous medium, higher concentrations of 8-cyclopentyl-dipropylxanthine could not be utilized for study. In contrast, a specific adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), completely reversed the antiinflammatory effect of methotrexate treatment (IC_{50} = 0.2 mg/kg, $P < 0.01$; Fig. 9) without affecting accumulation of leukocytes in either control animals (Fig. 5) or dexamethasone-treated animals (Fig. 7). We conclude from these experiments that the increased adenosine found at inflamed sites in methotrexate-treated animals mediates the antiinflammatory effects of methotrexate by engaging adenosine A_2 receptors.

Discussion

The results of the experiments reported here provide the first in vivo demonstration of a molecular mechanism for the antiphlogistic actions of methotrexate. Methotrexate, either acting directly or by promoting the intracellular accumulation of dihydrofolate polyglutamate, increases intracellular content of AICAR. The increase in intracellular AICAR concentration is associated with (and probably leads to) an increase in extracellular adenosine in inflammatory exudates. The increase in local adenosine concentrations at sites of inflammation suppresses inflammation via occupancy of adenosine A_2 receptors on inflammatory or connective tissue cells.

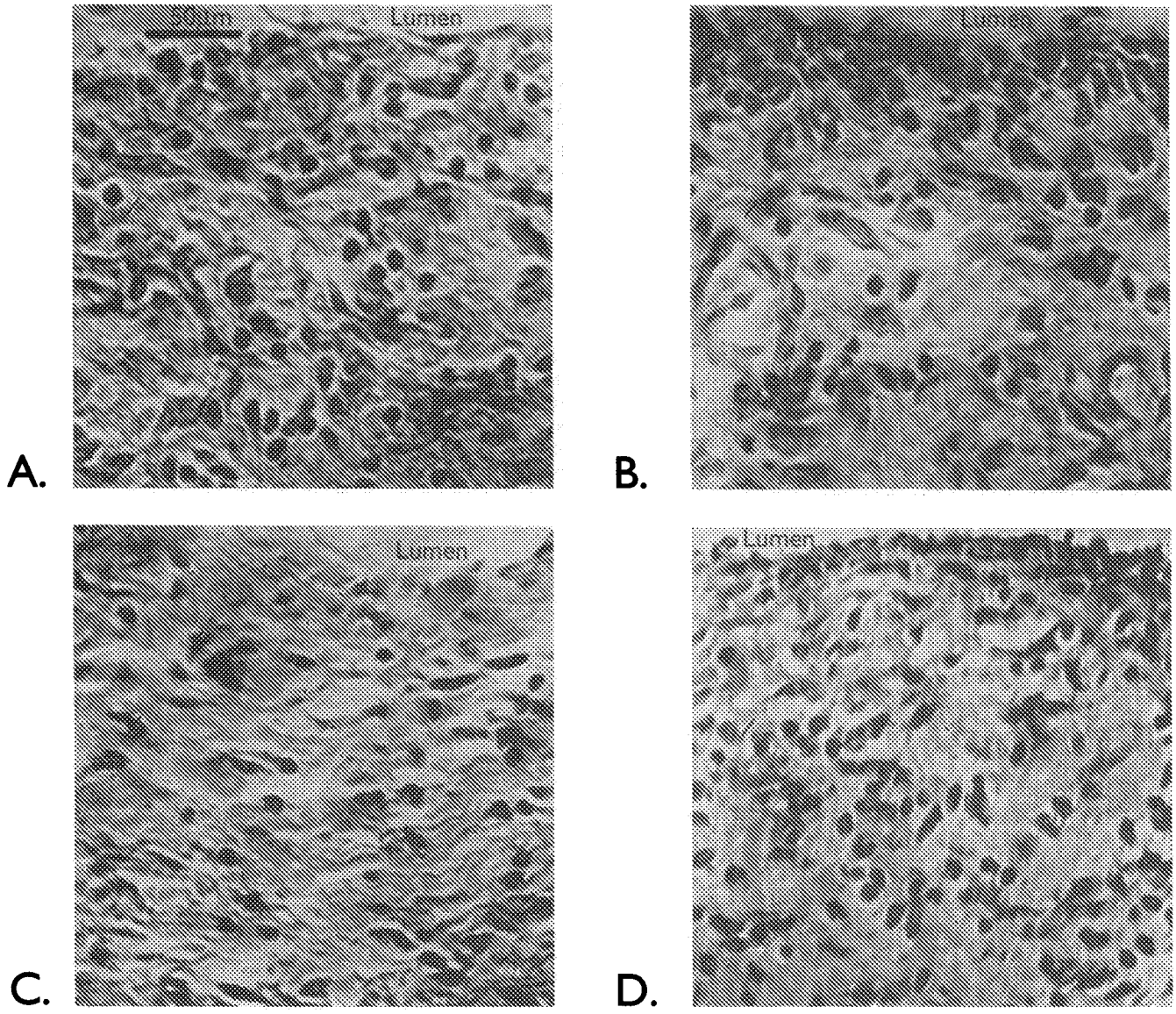

Figure 6. Adenosine deaminase (ADA, 0.15 IU/ml) reverses the antiinflammatory effects of methotrexate treatment (0.5 mg/kg per wk). Mice were treated with saline (control) or methotrexate for 3 wk before inflammation was induced in the air pouch. The air pouches were dissected out of the animals, and fixed and prepared by standard histopathological techniques for photomicroscopy. The photographic images were digitized directly using JAVA software and the images shown were adjusted only for brightness and contrast. Shown are representative fields (of 10 examined) from one section from one of two animals studied under each condition.

The observation that low-dose weekly methotrexate therapy promotes the intracellular accumulation of AICAR in splenocytes indicates that the "folate antagonism" of low-dose weekly methotrexate is highly specific. Via inhibition of dihydrofolate reductase, high concentrations of methotrexate diminish the cellular content of the methyl donors required for synthesis of purines and pyrimidines (6). In addition to the synthesis of formyl-AICAR from AICAR (Fig. 1), reduced folate is required for the synthesis of α-*N*-formylglycinamide ribonucleotide from β-glycinamide ribonucleotide, precursors of AICAR. Thus, under the conditions studied, if methotrexate inhibited folate-dependent reactions nonspecifically, then we would have expected either no change or a decrease in cellular AICAR content. We found the opposite, a net increase in cellular AICAR content, an observation that indicates that treatment with low concentrations of methotrexate leads to selective inhibition of AICAR transformylase without inhibiting the enzymatic steps required for the production of AICAR. The selective effect of low concentrations of methotrexate on purine biosynthesis most likely follows from the metabolism of methotrexate to its polyglutamated derivatives (for review see reference 6). Polyglutamated methotrexate directly inhibits several steps in the synthesis and metabolism of purines and pyrimidines (5, 7–9). In particular, polyglutamated methotrexate is a potent direct inhibitor of AICAR transformylase (7). Moreover, inhibition of dihydrofolate reductase by methotrexate (and methotrexate polyglutamate) leads to the intracellular accumulation of dihydrofolate polyglutamate, a known and potent inhibitor of AICAR transformylase (7–9). Since relatively high concentrations of methotrexate polygluta-

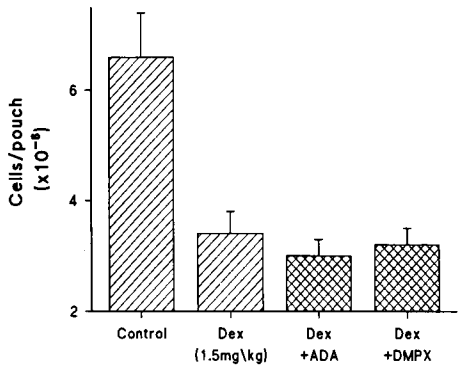

Figure 7. Neither adenosine deaminase (ADA, 0.15 IU/ml) nor DMPX (mg/kg) reverse the antiinflammatory effects of dexamethasone treatment (1.5 mg/kg). Air pouches were induced on mice for 3 wk. 1 h before injection of carrageenan into the air pouch, the mice received an intraperitoneal injection of dexamethasone (1.5 mg/kg) or saline. The exudates were harvested 4 h after injection of carrageenan and the cell number was quantitated. Dexamethasone significantly diminished the number of cells that accumulated in the air pouch and neither ADA nor DMPX significantly altered the number of cells that accumulated in the air pouch of animals treated with dexamethasone.

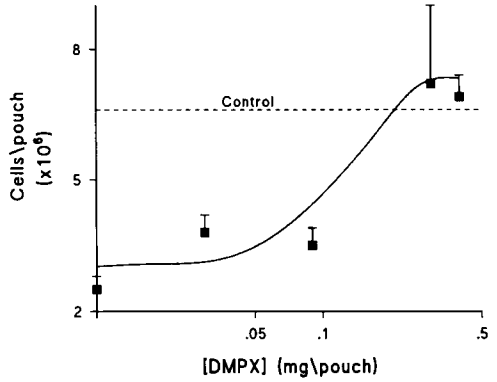

Figure 9. DMPX (mg/kg) reverses the antiinflammatory effect of methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates from three mice in the presence of the indicated concentrations of DMPX. Analysis of variance demonstrates that the number of cells in the pouch exudate varies significantly with the dose of DMPX ($P < 0.01$).

mates (7) are required to inhibit AICAR transformylase, it is more likely that dihydrofolate polyglutamates are responsible for the intracellular accumulation of AICAR. Nonetheless, treatment with methotrexate may lead to inhibition of AICAR transformylase (and accumulation of AICAR) by two different but complementary mechanisms.

Previous studies have demonstrated that intracellular accumulation of AICAR increases adenosine release from some, but not all, cell types (11). Barankiewicz et al. have shown that treatment of B-lymphoblasts with high concentrations of AICARibonucleoside diminishes adenosine uptake and utilization, resulting in increased release of adenosine into the extracellular space, particularly under conditions of ATP degradation (11, 19). In contrast to T lymphoblasts, which release little adenosine under any condition, B lymphoblasts possess increased AMP-5'-nucleotidase activity (adenosine formation) and relatively little adenosine kinase or adenosine deaminase activity (adenosine utilization [11]). Thus, Barankiewicz et al. postulated that, since AICARibonucleoside does not affect adenosine production or transport, intracellular accumulations of AICAR must inhibit adenosine kinase or adenosine deaminase activity in order to promote the increase in extracellular adenosine observed (11, 19). AICARibonucleoside may also lead to an increase in adenosine release at sites of "stress," such as reperfusion after ischemic insult to the heart, and the increased extracellular adenosine that accumulates in ischemic tissue protects the affected tissue from leukocyte-mediated injury (10). Our data suggest that treatment in vivo with low-dose methotrexate similarly increases intracellular AICAR content and, more importantly, promotes adenosine release at inflamed sites.

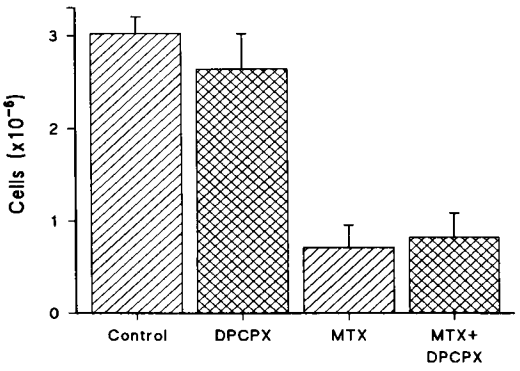

Figure 8. 8-Cyclopentyl-dipropylxanthine (DPCPX, 0.2 mg/kg) does not reverse the antiinflammatory effect of methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates from six mice in the presence of the indicated concentrations of DPCPX.

Methotrexate induced increased adenosine concentrations in inflammatory exudates and was a potent antiinflammatory agent in the air pouch model. To prove that the effects of methotrexate on purine metabolism and inflammation were causally related, we used two different approaches: elimination of extracellular adenosine by adenosine deaminase and antagonism of adenosine at its receptors with a specific antagonist (DMPX). Both of these experimental maneuvers reversed the antiinflammatory effect of methotrexate but did not reverse the antiinflammatory effect of dexamethasone in this same model. Dexamethasone is a potent agonist at glucocorticoid receptors that diminishes leukocyte accumulation at inflammatory sites by a mechanism that is not related to purine metabolism (for review see reference 20). Thus, our observation that both specific elimination and antagonism of adenosine reverse the antiinflammatory effects of methotrexate is strong evidence that adenosine mediates the antiphlogistic effect of methotrexate.

We have previously observed that methotrexate treatment, in vitro, promotes an increase in adenosine release at the expense of hypoxanthine and inosine release (4). In this study we were unable to detect inosine in most samples and the HPLC technique we used does not resolve hypoxanthine from many

other compounds present in these complex biologic fluids. Nevertheless, the adenosine concentration present in inflammatory exudates of methotrexate-treated animals (1.11 μM) is more than sufficient to account for the diminished inflammation observed; maximal inhibition of stimulated neutrophil adhesion and generation of superoxide anion and H_2O_2 is achieved with adenosine concentrations greater than or equal to 1 μM (14, 21). Indeed, the concentration of adenosine found in exudates from control animals was less than the concentration of adenosine found in transudates from "stressed" isolated rabbit hearts (during hypoxia, 1225±300 nM; reference 22). Although the adenosine concentration measured in the inflammatory exudate probably reflects the metabolic changes in methotrexate-treated animals and is sufficient to inhibit the production of toxic oxygen metabolites by the cells present in the inflammatory exudate, it is likely that the increase in extracellular adenosine responsible for diminished inflammation is that which occurs in the surrounding tissues, a less readily accessible site for sampling.

There are at least two major subclasses of adenosine receptor that can be distinguished on pharmacologic grounds, A_1 and A_2 (17, 18). Adenosine A_1 receptors are relatively high-affinity receptors that are linked to pertussis toxin-inhibited G proteins (23–33). Adenosine A_1 receptors have been demonstrated on neutrophils and macrophages (but not peripheral blood mononuclear cells) where they mediate, when occupied, enhanced chemotaxis and phagocytosis of immunoglobulin-coated particles (34–38). Adenosine A_2 receptors are low-affinity receptors linked to Gαs signal transduction proteins in many cell types. Adenosine A_2 receptors are present on neutrophils, monocytes, lymphocytes, and basophils and, when occupied, generally suppress the inflammatory or immune functions of these cells (for review see references 39–41). Using relatively selective antagonists we found that the antiinflammatory effects of adenosine in methotrexate-treated animals were mediated by occupancy of adenosine A_2 receptors, results that were identical to those obtained by Asako et al. (12). In contrast, Schrier et al. (42) observed, utilizing receptor-specific agonists, that occupancy of adenosine A_1 receptors rather than A_2 receptors is antiinflammatory in a rat model of inflammation. The discrepancy may be due to species differences in agonist sensitivity or adenosine receptor expression. Alternatively, the apparent difference in receptor specificity for the antiinflammatory effects of adenosine results from a difference in the distribution, lipid solubility, or other pharmacologic properties of the adenosine receptor-specific agonists studied.

We first suggested that adenosine might be an endogenous antiinflammatory agent when we observed that adenosine inhibits the generation of toxic oxygen metabolites by stimulated neutrophils (14). In subsequent studies we have shown that adenosine, both added exogenously or released endogenously, diminishes endothelial cell injury mediated by stimulated neutrophils (43). The cytoprotective effects of adenosine result from inhibition of the generation of toxic oxygen metabolites and inhibition of the stimulated adhesion of neutrophils to the endothelium (43). In the model under study, the apparent effect of adenosine was to diminish extravasation of leukocytes into an inflammatory exudate. There may be an additional beneficial effect of methotrexate therapy for the synovial tissues of patients treated with methotrexate; the concentration of adenosine present in the inflammatory pouch exudates is more than sufficient to inhibit generation of toxic oxygen metabolites by stimulated leukocytes. Thus, treatment with methotrexate may both diminish the number of leukocytes that accumulate in an inflammatory exudate and inhibit the destructive capacity of those leukocytes that do arrive at the inflamed site.

In the model studied here, inflammation was acute and was characterized by the accumulation of a neutrophilic infiltrate in both the air pouch and the surrounding tissues. Although it is likely that the adenosine released is acting directly on neutrophil adenosine receptors, it is also possible that adenosine inhibits the generation of cytokines or chemoattractants required for accumulation of the inflammatory exudate. Indeed, adenosine, probably acting at an A_2 receptor, inhibits synthesis of cytokines (TNFα) and other inflammatory proteins (complement C_2) by macrophages (44, 45). Moreover, adenosine, acting at its receptor, inhibits lymphocyte proliferation and induces suppressor activity in cultured lymphocytes (39). Thus, the antiinflammatory effects of methotrexate (acting via adenosine) are more general than those studied in this model of acute inflammation. Indeed, it is likely that the effects of methotrexate, acting via adenosine, on lymphocyte or monocyte function play a greater role in diminishing the chronic inflammation of rheumatoid arthritis than the effects on acute inflammation observed in this model.

We have demonstrated a novel biochemical mechanism of action of methotrexate. Low-dose weekly methotrexate therapy leads to intracellular accumulation of AICAR, which promotes increased adenosine release (and/or diminished adenosine uptake) at sites of inflammation. This increase in extracellular adenosine diminishes both the accumulation and function of leukocytes in inflamed sites. These findings suggest several novel approaches to the development of new agents that inhibit inflammation by increasing adenosine release: development of direct inhibitors of AICAR transformylase, inhibitors of adenosine deaminase and adenosine kinase, and adenosine uptake inhibitors.

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